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Patent and Trademark Office

To the Honorable Commissioner of Patents and Trademarks: Please record the attached original documents or copy thereof.

1. Name of conveying party: TRA AKTIEBOLAG Additional name(s) of conveying party(ies) attached? No	2. Name and address of receiving party: Name: **ALLELIX BIOPHARMACEUTICALS INC.** Internal Address: Street Address: **6850 Goreway Drive** City: **Mississauga, Ontario,** State: **Canada** ZIP: **L4V 1V7**
3. Nature of conveyance: **XX** Assignment __Merger __Security Agreement __Change of Name __Other Execution Date: **February 18, 1999**	Additional name(s) & address(es) attached? **No**

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)	B. Patent No.(s)
08/463,222, 07/806,271, 08/329,856, 07/239,145, 08/340,664, 08/461,436, 07/630,969	**5,646,015, 5,382,658, 5,208,041, 5,496,801, 5,223,407, 5,599,792**

Additional numbers attached? **No**

5. Name and address of party to whom correspondence concerning document should be mailed: Name: **Stephen A. Bent** Internal Address: **Suite 500** Street Address: **3000 K Street, N.W.** City: **Washington**, State: **DC** ZIP: **20007-5109**	6. Total number of applications and patents involved: **13**

	7. Total fee (37 C.F.R. §3.41). . . . . . .\$**520.00**
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9. Statement and signature.
To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Stephen A. Bent	Signature	March 22, 1999
Name of Person Signing Reg. No. **29,768**		Date

Total number of pages including cover sheet, attachments, and document: _4

<u>ASSIGNMENT</u>

For good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, the undersigned company:

ASTRA AKTIEBOLAG

having a place of business at

S-151 85 Soderalje
Sweden

owner of the invention rights in the United States of America and its territories and possessions to the below listed U.S. Patent, as evidenced in the Assignment records in the U.S. Patent and Trademark Office, and by these presents hereby sells and assigns, unto:

ALLELIX BIOPHARMACEUTICALS INC.

having an address at

6850 Goreway Drive
Mississauga, Ontario
Canada L4V 1V7

(hereinafter ASSIGNEE) all right, title and interest for United States, its territories and possessions thereof, in and to the inventions and the applications therefor, identified as follows:

PATENT NUMBER	ISSUE DATE	TITLE
H 1606-3 US US 5,646,015	July 8 1997	EXCRETION OF HETEROLOGOUS PROTEINS FROM *E. COLI*
H 1603-1 US US 5,382,658	January 17, 1995	STABILITY - ENHANCED VARIANTS OF PARATHYROID HORMONE
H 1601-1 US US 5,208,041	May 4, 1993	ESSENTIALLY PURE HUMAN PARATHYROID HORMONE
H 1615-1 US US 5,496,801	May 5, 1996	PARATHYROID HORMONE FORMULATION
H 1606-2 US US 5,223,407	June 29, 1993	EXCRETION OF HETEROLOGOUS PROTEINS FROM *E. COLI*

US 5,599,792   February 4, 1997   BONE-STIMULATING, NON-VASOACTIVE PARATHYROID HORMONE VARIANTS

Serial No.	Filing Date	TITLE
H 1608-8 US 08/463,222	June 5, 1995	PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICTOORGANISMS
H 1602-2 US 07/806,271	December 13, 1991	OXIDATION RESISTANT VARIANTS OF PARATHYROID HORMONE
H 1603-2 US 08/329,856	October 27, 1994	STABILITY ENHANCED VARIANTS OF PARATHYROID HORMONE
H 1606-1 US 07/239,145	August 31, 1988	EXCRETION OF HETEROLOGOUS PROTEIN FROM *E. COLI*
H 1608-6 US 08/340,664	November 16, 1994	PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS
H 1608-7 US 08/461,436	June 5, 1995	PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICOORGANISMS
H 1602 · US 07/630,969	December 20, 1990	PARATHYROID HORMONE VARIANTS

including all reissue and extensions of Letters Patent granted for said invention; the Commissioner of Patents and Trademarks of the United States Of America being hereby authorized to transfer said Letters Patent to said Assignee in accordance herewith; this assignment being under covenant, not only that full power to make the same is had by the undersigned, but also that such assigned right is not encumbered by any grant, license, or other right heretofore given, such that the Letters Patent shall be held and enjoyed by said Assignee as fully and entirely as the same could have been held and enjoyed by the undersigned if this assignment had not been made.

The undersigned hereby grants the firm of FOLEY & LARDNER the power to insert in this Assignment any further identification which may be necessary or desirable to comply with the rules of the U.S. Patent and Trademark Office for recordation of this Assignment.

Company:
Astra Aktiebolag
(publ)

Date: _____18.02.1999_____   By: _____(signature)_____
                                              Name: p.p. Claes Wilhelmsson
                                              Title: Executive Vice President
                                                     Research & Development

Date: _____18.02.1999_____   WITNESS: _____(signature)_____

Date: _____18.02.1999_____   WITNESS: _____(signature)_____

Note: **Prima** **facie** evidence of execution may optionally be obtained by execution of this document before a U.S. Consul or before a local officer authorized to administer oaths whose authority is proved by certificate from a U.S. Consul.

APPENDIX 2

CLAIMS ON APPEAL PRIOR TO AFTER–FINAL AMENDMENT

31.   hPTH (1-84), as a substantially homogeneous protein.

32.   hPTH (1-84) having a purity of greater than 95%.

33.   hPTH (1-84), as a substantially homogeneous protein, prepared by a process comprising the steps of:

providing a microorganism containing exogeneous DNA encoding hPTH (1-84);

culturing said microorganism to allow expression of said exogeneous DNA, thereby producing hPTH (1-84); and

purifying said hPTH (1-84) as a substantially homogeneous protein.

34.   The substantially homogeneous hPTH (1-84) of claim 33, wherein said microorganism is *E. coli*.

35.   The substantially homogeneous hPTH (1-84) of claim 33, wherein said microorganism is yeast.

APPENDIX 3

CLAIMS ON APPEAL AFTER ENTRY OF THE AFTER-FINAL AMENDMENT

31.   A substantially homogeneous recombinant human parathyroid hormone (hPTH (1-84)) protein.

32.   A recombinant human parathyroid hormone (hPTH (1-84)) having a purity of greater than 95%.

33.   A substantially homogeneous recombinant human parathyroid hormone (hPTH (1-84)) protein, prepared by a process comprising the steps of:

(a) providing a microorganism containing exogeneous DNA encoding hPTH (1-84);

(b) culturing said microorganism to allow expression of said exogeneous DNA, thereby producing hPTH (1-84); and

(c) purifying said hPTH (1-84) as a substantially homogeneous protein.

34.   The substantially homogeneous hPTH (1-84) of claim 33, wherein said microorganism is *E. coli*.

35.   The substantially homogeneous hPTH (1-84) of claim 33, wherein said microorganism is yeast.

PATENT
GAUTVIK 3.0-001 FWC CIP CONT FWC DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of
   Gautvik et al..

Serial No. 08/340,664

Filed: November 16, 1994

For: PRODUCTION OF HUMAN
    PARATHYROID HORMONE FROM
    MICROORGANISMS

: Group Art Unit: 1812

: Examiner: L. Spector

Assistant Commissioner for Patents
Washington, D. C. 20231

DECLARATION OF JOHN E. MAGGIO, Ph.D. PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, John E. Maggio, declare as follows:

1. I am a citizen of the United States of America residing at 480 Washington Street, Brookline, Mass. 02146.

2. I am an Associate Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School, Boston, Massachusetts. I have been a professor at Harvard Medical School since 1985.

3. My formal education includes Bachelors, Masters and Doctoral degrees, in the fields of chemistry and organic chemistry, all from Harvard University. I have also had extensive post-doctoral training at the University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge University, Cambridge, England, and at the Neuropsychopharmacology Research Unit at Yale University Medical School in New Haven. Both post-doctoral positions involved protein and peptide chemistry and purification thereof. My current curriculum vitae, including a list of my publications is attached as Exhibit 1.

4. My past and present work centers around synthesis, purification and characterization of biologically active peptides including tachykinins, magainins and amyloid peptides. As part of my work and since at least the late 1970s, I have used various forms of chromatography and electrophoresis for the purification of peptides and for their characterization. I am fully familiar with these techniques, and the state of their development throughout the 1980s and 1990s. I am also fully familiar with the past and present capabilities and limitations of such techniques. Representative of my work with peptide separation and characterization is an article attached as Exhibit 2 entitled "Mapping Peptide-binding Domains of the Substance P(NK-1) Receptor from $P388D_1$ Cells with Photolabile Agonists", *J. Biological Chemistry*. 270, (1995), 1213-1220.

5. My knowledge of chromatography, electrophoresis, and other techniques used commonly in protein chemistry stems from my repeated use of those techniques throughout my career. I have supervised students and other scientists using these techniques and have taught the techniques, both in the classroom and in the laboratory. Therefore, I am comfortable judging the ordinary level of skill that a person in this art would possess in terms of the theoretical and the bench aspects of these techniques.

6. In preparing this declaration I reviewed, among other things, the following materials: a copy of the specification of U.S. Serial No. 08/340,664, filed November 16, 1994, (the "'664 application"); a copy of the Official Action dated September 8, 1995 issued by the patent examiner, Dr. Spector; copies of each of the references identified in the

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Official Action; and a copy of the Declaration of Kaare M. Gautvik, M.D. Pursuant to 37 C.F.R. § 1.132 as well as the documents and photographs attached thereto.

7. I understand from my review of the Official Action, the United States Patent and Trademark Office has refused to grant the '664 application in view of the disclosures of four references: *Brewer et al., Fairwell et al., Kimura et al.* and/or *Kumagaye et al.*

8. I have reviewed the four references cited by the Patent Office and I do not agree with the Patent Office's conclusions regarding their teachings or disclosures. In my opinion, none of the references describe or suggest a method of obtaining a substantially pure, intact, hPTH peptide. None of the references provides a basis for concluding that a substantially pure hPTH product was actually produced. Further, nothing in the references describes an hPTH peptide having biological activity substantially equivalent to naturally occurring hPTH. I believe that a biochemist, organic chemist or analytical chemist having an ordinary level of skill in this technology would not be unable to draw any conclusion with regard to the purity of hPTH produced in accordance with the cited references. If anything, given the errors appearing in those references and the known shortcomings of the techniques described in the references, *e.g.* solid phase chemical synthesis, those of ordinary skill in the art would probably assume that the resulting hPTH material was impure.

9. *Brewer et al.* relate to an isolation from tissue, not a recombinant material. *Brewer et al.* contain three errors at positions 22, 28 and 30 of the synthesized peptide compared to the wild-type peptide. This is illustrated

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in Fig. 1 of *Brewer et al.* Accordingly, *Brewer et al.* do not teach the production of an *intact* hPTH peptide. Moreover, two later publications cited by Dr. Spector in the Official Action, namely *Kimura et al.* and *Kumagaye et al.*, show that the purification protocols discussed in *Brewer et al.* result in impure materials. For example, Fig. 2, on page 496 of *Kimura et al.* is an HPLC profile of crude product obtained after use of a separation protocol analogous to that disclosed in *Brewer et al.*; namely, the use of a combination of gel filtration and ion exchange chromatography. Impurities are plainly evident. Therefore, a conclusion of homogeneity based on *Brewer et al.* is unjustified. Further, *Kimura et al.* describe a purification sequence of CM-cellulose column chromatography followed by gel filtration on Sephadex G-50, followed in turn by the use of reverse phase-high pressure liquid chromatography ("RP-HPLC"). *Kimura et al.* added the RP-HPLC step in recognition of the need to obtain better purity than *Brewer et al.* obtained. This fact alone, in my opinion, eliminates any plausible basis for concluding that the protein resulting from the methods described in *Brewer et al.* was essentially pure.

10. Many of the criticisms of *Brewer et al.* apply to *Fairwell et al.* For example, *Fairwell et al.* produced a peptide having an Asp at position 76. Native hPTH has an Asn in that position. *Fairwell et al.* also used a separation protocol combining the use of gel filtration and ion exchange chromatography. As previously mentioned, that protocol was re-run by *Kimura et al.* and the results, as illustrated in the chromatogram in Fig. 2 thereof, show significant impurities. Finally, as *Kimura et al.* used RP-HPLC rather than relying

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merely on the separation protocol described in *Fairwell et al.*, it is clear that subsequent investigators believed that the separation protocols of *Fairwell et al.* were inadequate.

11. *Kimura et al.* did not produce an essentially pure hPTH. As *Kumagaye et al.* clearly explain, "[t]oday, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products." (Emphasis added) *Kumagaye et al.* at page 330. This is especially significant because *Kumagaye et al.* is the same group of researchers as *Kimura et al.*

12. *Kumagaye et al.* disclose a method of separating two different forms of hPTH from a mixture thereof by using cation exchange-HPLC. This is not a particularly surprising result as the two forms of hPTH disclosed have a full charge difference between them, a situation ideal for the use of cation exchange-HPLC. Nonetheless, one of ordinary skill in the art would not conclude that the resulting hPTH in accordance with *Kumagaye et al.* was essentially pure. One could conclude that the resulting material was pure of the one specific impurity, i.e. the specific point mutated form disclosed. However, there is no basis for concluding further. If anything, as explained herein, there is every reason to believe that impurities are present.

13. *Kumagaye et al.* describe a solid phase peptide synthesis protocol which was common at the time. That synthesis, as explained in the *Kimura et al.* article, involved the use of BOC protected amino acids and traditional BOC chemistry. Using BOC chemistry, each successive amino acid is

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added to the N-terminus of a growing chain by first removing the blocking group in acid, then neutralizing prior to coupling, followed by coupling the next BOC amino acid in sequence using, for example, dicyclohexylcarbodiimide ("DCC").

14. This technology suffers from a number of well known shortcomings and, in fact, has largely been replaced. One of the better known and most common problems with solid phase BOC chemistry, particularly for longer peptides such as hPTH, is racemization. As explained in *Bodanszky,* "Peptide Chemistry; A Practical Textbook" at page 120, the problem of racemization using DCC coupling and BOC chemistry is well documented. *See* Exhibit 3. This chapter, as well as the others attached as Exhibit 3, demonstrate the prevalence of racemization and concerns over this phenomenon during solid phase protein synthesis.

15. Some of the other well known impurities generated by solid phase synthesis are described in the *Fairwell et al.* article cited by Dr. Spector at page 2691. These impurities include, among other things, deletion peptides, omission peptides and prematurely terminated peptides. For example, during solid phase synthesis it is possible for coupling to be either duplicative or incomplete, thereby providing a peptide having an additional amino acid or an omission from the normal sequence. There may be one or more additions and/or deletions in any given peptide. These additions and/or deletions can occur almost anywhere along the chain. Premature termination of the chain length is also common. This may occur for a host of reasons such as, peptides folding in on themselves, side reactions to make the N-terminal amino acid unavailable for

further coupling, steric hindrance, premature cleavage from the bead, and the like.

16. To fully understand the magnitude of the purification problems presented by the use of this type of synthetic chemistry, one needs to consider that all three of the foregoing problems, incomplete coupling, premature termination, and racemization, are occurring simultaneously. The result often is a wide variety of incorrect peptides, frequently including two or more of the aforementioned errors. The frequency of these errors, and therefore the degree of impurity, increases exponentially with the length of desired peptide. Proteins such as hPTH, which is 84 amino acids in length, are considered to be long and difficult to make synthetically, even by today's sophisticated standards. The technique employed by *Kumagaye et al*, cation exchange-HPLC, could be used for separating some of the resulting impurities. However, this technique would only work for that fraction of the total impurities having a charge differential when compared to native hPTH; a relatively minor percentage of the total impurities. In addition, depending upon the conditions used, not all of the differently charged species will be separated. Some of the resulting impurities may have a charge which is very similar to native hPTH, and may co-elute with hPTH. Any single impurity, if known, could theoretically be removed from the mixture by HPLC, RP-HPLC, and/or some other separation technique(s). Here, with the many possible impurities, it would be nearly impossible to effectively remove them all.

17. Persons familiar with cation exchange-HPLC would realize that the types of impurities to which I have referred result, almost inevitably, from the use of solid phase BOC

chemistry and that many of the impurities co-elute with intact hPTH. Therefore, one would conclude, as I concluded, that the hPTH material resulting from the protocol described in *Kumagaye et al.* is pure only insofar as the one disclosed point mutant. No further conclusions about purity can be made. If anything, the impurities known to result from the solid phase synthesis described in the references would lead to the conclusion that the hPTH resulting from *Kumagaye et al.* would contain other impurities. *Kumagaye et al.* provide no explicit recitations of purity, provide no other form of characterization of the quality or quantity of the resulting hPTH material and provide nothing with regard to biological activity. Due to the cellular editing mechanisms found in, for example, yeast and *E. coli*, such impurities would not occur.

18. My opinion of all of the references, and, in particular *Kumagaye et al.,* is strengthened by the comparisons that I have reviewed between recombinant hPTH manufactured as described in the '664 application and commercially available, synthetically produced, hPTH produced by solid phase peptide synthesis.

19. As stated in paragraph 6, I have reviewed the declaration of Dr. Kaare M. Gautvik and, in particular, the photographs labeled Glossy 0 through Glossy III attached in Exhibits B-E, respectively, thereto. I understand from Dr. Gautvik's declaration that the materials analyzed and depicted in these photographs were made pursuant to the techniques described in the '664 application. Having reviewed that specification, I have no reason to question that assertion. The photographs are particularly informative because they provide a direct comparison between peptides produced by solid

phase chemical synthesis and recombinant technology as described in the '664 application.

20. Glossy 0 illustrates an electrophoretic gel comparing recombinantly produced hPTH from Dr. Gautvik's laboratory with hPTH produced by solid phase synthesis sold by Sigma. Lane 2 (second from the left) contains the recombinant hPTH produced by Dr. Gautvik. The single broad band indicates homogeneity. In contrast, the Sigma material illustrated in Lane 3 shows a band migrating at roughly the same position as the hPTH produced recombinantly and two additional impurities of higher molecular weight. Based on the presence of these impurities in the Sigma material, the intensity and breadth of the bands and the relative intensities and sizes of the bands of hPTH, it is not hard to see that the recombinant material is orders of magnitude purer than the Sigma material.

21. Glossy III shows molecular weight standards in Lane S as well as recombinant hPTH produced from *E. coli* (Lane 4) and yeast (Lane 2) produced in accordance with -the procedures outlined in the '664 application. Disposed between these materials, in Lane 3 is a synthetic material produced by solid phase synthesis available from a second chemical supplier, Bachem. The recombinant material is characterized by a single, sharp, dark, broad band corresponding to hPTH. In contrast, the Bachem lane illustrates the presence of lower molecular weight impurities in a smear. Moreover, the difference in the intensity of the staining indicates a significantly greater amount of hPTH in the recombinant preparations than in the chemically synthesized preparations, using an identical load (800 nanograms) of assayed material.

22. Glossy II contains, in addition to the information illustrated in Glossy III (lanes 27 through 29), identical preparations at a loading of 200 nanograms, (lanes 22 through 24). The difference in the intensities of the bands between the recombinantly produced material and the solid phase synthetic material available from Bachem illustrates the significantly greater amount of hPTH in the recombinant material, per unit weight. (Lanes 22 and 24 contain recombinant material and line 23 contains synthetic.) This information is totally consistent with the HPLC, N-terminal amino acid sequencing, mass spectrometry and two dimensional gel electrophoresis described in the '664 application. Based on this information, one of any level of skill in this art would conclude, as I have, that the recombinant material produced in accordance with the present invention is essentially pure.

23. My opinions are both verified and amplified by my review of Dr. Gautvik's declaration and, in particular, his publication in the peer-refereed journal *Peptides*, attached to his Declaration as Exhibit F. This article clearly demonstrates the biological properties of the recombinant material produced in accordance with the '664 application and verifies that which would be implicitly understood therefrom; namely that the hPTH material of the invention has biological activity substantially equivalent to naturally occurring human parathyroid hormone. Chemically synthesized material does not.

24. Dr. Gautvik's *Peptides* article is significant in that it illustrates both *in vivo* and *in vitro* biological activity. The results consistently reemphasize the superiority of the recombinant hPTH material in direct side-by-side comparisons to synthetic material. Fig. 1 of the *Peptides*

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paper illustrates the differences in binding affinity between recombinant hPTH produced in accordance with the '664 application from both *E. coli* and yeast and synthetic material from Bachem. The K_d of the recombinant material was 9.5nM while the K_d of the Bachem material was 18nM. This illustrates that the recombinant material contains approximately twice as much authentic hPTH when compared to the chemically synthesized material. The differences between these K_d values are very statistically significant as described in the paper (95% confidence intervals and redundant testing in triplicate). Because the K_d values indicate a greater amount of authentic hPTH per unit weight, the significant difference between the two K_d values indicate a dramatic difference in purity.

25. Fig. 2 of the *Peptides* paper illustrates the abilities of different preparations of hPTH to elicit a biological response in cell cultures. From Fig. 2 one can determine both an EC_{50} for cyclic AMP (cAMP) as well as a measure of efficacy or maximal response. EC_{50} is a measure of the potency of the materials in question. Here, the EC_{50} for recombinant hPTH is 1.5nM. The Ec_{50} for the Bachem material was 5.7nM. This is almost a four fold difference. As the figure and the accompanying text illustrate, this difference is highly statistically significant.

26. One of the more surprising findings outlined in the *Peptides* paper is the efficacy of the resulting materials. It appears that the Bachem material is only about 70% as efficacious as the recombinant hPTH. This means that no dose of synthetic material would be able to produce the maximal response of the tested cells, a problem not shared by the recombinant material. These two figures, acting in

combination, illustrate that the recombinant material is superior not only in purity, but also in binding and in eliciting a biological response. Moreover, the data just described are completely consistent with the data illustrated in Figs. 3-5 which show the *in vivo* activity of recombined hPTH in rats. In particular, Fig. 3 confirms the efficacy and potency data described in Fig. 2 by exhibiting an increased level of blood calcium over that achievable through the use of the synthetic material. Even 2.7 micrograms of Bachem PTH was unable to produce the same results as 2.0 micrograms of recombinant PTH.

27. The *Peptide* paper is a particularly good comparison of the recombinant material produced in accordance with the '664 application and synthetically produced material because of its careful characterizations and the variety of analytical techniques used. For example, peptide concentrations were determined by amino acid analysis. This is the premier method of determining peptide concentrations to date and is superior to other techniques such as optical density or dry weight. *In vivo* activity was measured not in one system, but rather by induction of hypercalcemia, urinary excretion of phosphate and by changes in urinary cAMP after administration of hPTH. Moreover, *in vitro* activity was assayed by receptor binding and cAMP responses of cells in culture. The variety of techniques used would appear to conclusively establish the superiority of recombinant material over synthetic material, both biologically and in terms of its purity. For these reasons, I believe that essentially pure recombinant material results from the practice of the invention described in the above-captioned application and that this hPTH

material is superior to anything in the prior art. I also believe that one of ordinary skill in the art would, upon reading the application, conclude as I have.

28. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: <u>6 MARCH 1996</u>

<u>JOHN E. MAGGIO, Ph.D.</u>

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-13-

CURRICULUM VITAE

NAME	John E. Maggio
ADDRESS:	Department of Biological Chemistry and Molecular Pharmacology Harvard Medical School 240 Longwood Avenue Boston, MA 02115
DATE OF BIRTH:	November 6, 1952
PLACE OF BIRTH:	Houston, Texas

EDUCATION:

1975	A.B.	Harvard College, Cambridge, MA (Chemistry)
1975	A.M.	Harvard University, Cambridge, MA (Chemistry)
1981	Ph.D.	Harvard University, Cambridge, MA (Organic Chemistry)

POSTDOCTORAL TRAINING:

1981-83	Postdoctoral Research Associate, University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge, England
1984-85	Postdoctoral Fellow, Neuropsychopharmacology Research Unit, Yale University School of Medicine, New Haven, CT

ACADEMIC APPOINTMENTS:

1985-87	Assistant Professor of Pharmacology, Harvard Medical School, Boston, MA
1987-91	Assistant Professor of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA
1991-	Associate Professor of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

AWARDS AND HONORS:

1971-75	Dean's List, Harvard College Scholarship for Outstanding Academic Achievement, National Merit Scholar
1975	A.B. *magna cum laude* with Highest Honors
1976-78	National Science Foundation Graduate Fellow
1981-82	Member of the High Table, King's College, Cambridge, England
1981-82	North Atlantic Treaty Organization / National Science Foundation Postdoctoral Fellow
1983-84	Muscular Dystrophy Association Postdoctoral Fellow

ORIGINAL PUBLICATIONS:

1. Maggio, J.E.: Structure of a mycobacterial polysaccharide - fatty acyl-CoA complex: Nuclear magnetic resonance studies. Proc. Natl. Acad. Sci. USA 77: 2582-2586, 1980.

2. Simmons III, H.E., and Maggio, J.E.: Synthesis of the first topologically nonplanar molecule. Tetrahedr. Lett. 22: 287-290, 1981.

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4. Benner, S.A., Maggio, J.E., and Simmons, H.E., III: Rearrangement of a geometrically restricted triepoxide to the first topologically nonplanar molecule: A reaction path elucidated by using oxygen isotope effects on carbon-13 chemical shifts. J. Am. Chem. Soc. 103: 1581-1582, 1981.

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6. Welzel, P., Wietfeld, B., Kunisch, F., Schubert, T., Hobert, K., Duddeck, H., Muller, D., Huber, G., Maggio, J.E., and Williams, D.H.: Moenomycin A: Further structural studies and preparation of simple derivatives. Tetradedr. Lett. 39: 1583-1591, 1983.

7. Sandberg, B.E.B., Maggio, J.E., Bishai, W.R., and Hannah, P.A.: A conformational approach to structure-activity studies of substance P. In: Substance P - Dublin 1983 (Skrabanek, P., and Powell, D., Eds.), Boole Press, Dublin, pp. 18-19, 1983.

8. Maggio, J.E., Sandberg, B.E.B., Bradley, C.V., Iversen, L.L., Santikarn, S., Williams, D.H., Hunter, J.C., and Hanley, M.R.: Substance K: A novel tachykinin in mammalian spinal cord. In: Substance P - Dublin 1983 (Skrabanek, P., and Powell, D., Eds.), Boole Press, Dublin, pp. 20-21, 1983.

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10. Hunter, J.C., Maggio, J.E., and Mantyh, P.W.: Evidence for vasoactive intestinal polypeptide as a neurotransmitter in smooth muscle of the urogenital tract. Brain Res. 305: 221-229, 1984.

11. Mantyh, P.W., Hunt, S.P., and Maggio, J.E.: Substance P receptors: Localization by light microscopic autoradiography in rat brain using ^{3}H-SP as the radioligand. Brain Res. 307: 147-166, 1984.

12. Mantyh, P.W., Maggio, J.E., and Hunt, S.P.: The autoradiographic distribution of kassinin and substance K binding sites is different from the distribution of substance P binding sites in rat brain. Eur. J. Pharmacol. 102: 361-364, 1984.

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conference, Amyloid and other abnormal protein assembly processes, August, 1995.

AFFILIATIONS:
American Chemical Society
Society for Neuroscience
International Brain Research Organization
American Association for the Advancement of Science
Foundation for Biomedical Research
American Peptide Society
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North Atlantic Treaty Organization (1982)
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Milton Fund of Harvard University (1989) (1994)
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Endocrinology (Associate, Executive Committee)
Neuroscience (Associate, Admissions, Appointments, Steering Committee)
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Neurological Sciences Academic Development (Associate)
Biological Sciences in Public Health (Associate)

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Dr. Maggio has served on and chaired a wide range of departmental, program, medical school, and university committees, such as: Faculty Search, Admissions, Facilities, Thesis Advisory, Qualifying/Preliminary Examination, Prize, Curriculum, Thesis Defense, Course Planning, Steering, Executive, Criteria, Etc. *See Service.*

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Biological Chemistry and Molecular Pharmacology (graduate)*
Conduct of Science (graduate)
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　AIDS and Related Research (ARR-5, 12/88)
　Neurological Sciences (NLS-1, 6/90, 10/90, 6/91, 10/91, 10/92, 10/93, 10/94)
　Neurology (NEUB-1, 6/95)

NSF Applications (ad hoc)

Journals (ad hoc):
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Dr. Maggio's graduate work included research on noncovalent interactions, organic synthesis, reaction mechanisms, biological control, NMR spectroscopy, and biochemistry under the mentorship of Jean-Marie Lehn, Robert B. Woodward, and (principally) Konrad E. Bloch. He received the Ph.D. in Organic Chemistry from Harvard University in 1981. His postdoctoral research on various aspects of neuropeptides and neuropeptide receptors was carried out at the Medical Research Council and the University of Cambridge, UK, with Leslie L. Iversen and Dudley H. Williams; and later at Yale University School of Medicine with Robert H. Roth. He joined the faculty of Harvard Medical School in 1985, and is presently Associate Professor of Biological Chemistry and Molecular Pharmacology.

RESEARCH INTERESTS

The bioactive peptides are the largest and least understood class of intercellular messengers, carrying out a diverse set of functions in a wide variety of systems. Understanding bioactive peptides and their receptors, in the nervous system and elsewhere, is the general research goal in our group.

One system of interest is the tachykinin (substance P) family of peptides and receptors, which are involved in transmission of primary afferents and thus in pain and neurogenic inflammation. As the primary structures of both the ligands and their receptors are known, an excellent model system for peptide-protein interactions in signalling is available. Recently we have identified through photoaffinity labelling which regions of the peptide substance P interact with which regions of its G-protein-coupled receptor, a protein whose expression is upregulated a thousand-fold in some inflammatory diseases. Radioactive, fluorescent, and antibody probes of these receptors allow studies of desensitization and internalization *in vivo* and *in vitro*.

Another system under investigation is the process of amyloid formation in Alzheimer's disease (AD) and other amyloidoses. The characteristic lesion of AD is brain senile plaques formed mainly of the human amyloid peptide Aß, a ≈40-mer which occurs naturally in normal as well as AD brain. By reconstituting plaque growth (deposition of Aß at physiological concentrations onto authentic plaques) *in vitro*, we can characterize the process and identify conditions and components which enhance or inhibit its kinetics. Structure/activity studies have identified amino acids critical for amyloid deposition and active peptide analogues suitable for high resolution structure determination by nuclear magnetic resonance spectroscopy. The latter studies have further identified conformational elements essential to plaque deposition.

Another interest is the characterization of novel bioactive peptides from natural sources. A particularly rich source is the skin venom of certain neotropical frogs. The peptides found here include antibiotics and toxins as well as close analogs of discovered and yet undiscovered mammalian neuropeptides.

REFERENCES available on request.

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Miklos Bodanszky

Peptide Chemistry

A Practical Textbook

Second, Revised Edition

With 2 Figures and 5 Tables

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To the memory of
my brother Dr. S. Bodanszky

The title illustration shows a section of a peptide in van-der-Waals representation of the atoms. It was generated with the modelling program MOBY by U. Hüweler, available from Springer-Verlag.

VIII. Racemization

...be applied in an excess that provides for a concentration of at least 10^{-1} M throughout the coupling reaction. This measure (the "principle of excess") counteracts the decrease in rate which necessarily occurs in bimolecular reactions as the concentration of the reactants decreases. Therefore the extent of unimolecular side reactions, in which the rate is independent of concentration, can be markedly reduced. An excess of acylating agent also helps to achieve complete acylation of the amine-component and prevents thereby the formation of "deletion sequences", peptides from which one amino acid residue is missing.

A further concentration related problem has to be mentioned here. Following deprotection by acidolysis the regenerated amine is isolated as a salt of the acid used for cleavage. In the subsequent acylation step, however, the free amine is needed. Deprotonation with the help of ion exchangers can be applied or, in solid phase peptide synthesis (Chapter X) treatment with a tertiary amine and removal of the trialkylammonium salts by washing. In syntheses carried out in solution the general practice is to "liberate" the amine-component from its salt by adding an equimolar amount of a tertiary amine (triethylamine, diisopropylethylamine, N-methylmorpholine or N-ethylpiperidine) to the reaction mixture prior to coupling. It is obvious, however, that often only an equilibrium

$$R-NH_3^+ + R_3'N \rightleftharpoons R-NH_2 + R_3'NH^+$$

can be established. While it is true that during acylation of the amino group this equilibrium is gradually shifted to the right, at any given time the concentration of the amine-component is lower than it would be if applied in completely deprotonated form. A notable exception is created by the insolubility of tertiary ammonium salts in certain solvents. For instance triethylamine hydrochloride, being practically insoluble in ethyl acetate, separates from the reaction mixture (if ethyl acetate is used as solvent for coupling) and shifts the equilibrium in the desired direction. The presence of tertiary amines is usually unfavorable during coupling: they can initiate side reactions through proton abstraction. These reactions can be suppressed by the addition of certain weak acids, for instance 2,4-dinitrophenol or pentachlorophenol, which show a distinct affinity for tertiary amines. They do not protonate the amine-component firmly enough to prevent its acylation. Moreover, the application of tertiary amines can be avoided by selecting highly acid sensitive amine-blocking groups and removing them with suitable weak acids, such as 1-hydroxybenzotriazole or tetrazole. The resulting salts are readily acylated, even with moderately reactive esters, without the addition of a tertiary base.
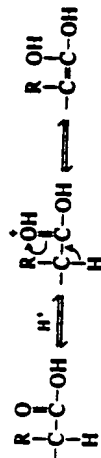
Additional Source

Bodanszky, M., Martinez, J.: Side Reactions in Peptide Synthesis, in The Peptides, vol. 5, Gross, E., Meienhofer, J. eds., pp. 111-216, New York, Academic Press 1983
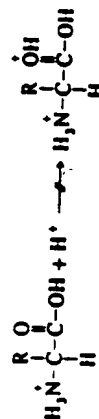
With the exception of glycine, in all amino acids that are constituents of proteins, the α-carbon atom is chiral. In threonine and isoleucine a chiral center is present in the side chain as well. In order to secure the target peptide in homogeneous form it is absolutely essential to start from enantiomerically pure amino acids and to insist on conservation of chiral homogeneity throughout the various operations of synthesis. Otherwise, instead of a single product, a mixture of stereoisomers will be obtained. Their number in a peptide with n chiral centers is 2^n. Accordingly, if racemization is not prevented, even in the synthesis of a moderately large peptide a complex mixture will be produced and separation of the desired material from a multitude of similar compounds might turn out to be an at least arduous and sometimes overwhelming task. Therefore, the importance of racemization studies and of the measures that must be taken for the prevention of any loss in chiral purity can not be overemphasized. In fact, "strategies" of peptide synthesis, that is general planning of schemes for syntheses (Chapter IX) are dictated primarily by considerations concerning conservation of chiral homogeneity.
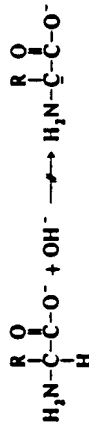
A. Mechanism of Racemization

With respect to chiral stability amino acids are fairly insensitive to acids and bases. Racemization via enolization of carboxylic acids in acidic solutions involves protonation of the carbonyl oxygen

yet, the presence of a nearby positively charged nitrogen atom hinders the formation of the second cation:
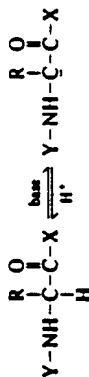
that was first postulated and subsequently proven by ir spectra. Azlactone formation is quite pronounced in benzoylamino acids, less prevalent in acetyl-amino acids and was for a long time thought to be absent in amino acids acylated by benzyloxycarbonyl or other urethane type blocking groups. The absence of racemization on activation of the latter was attributed to lack of azlactone formation, but in recent years azlactones were obtained from benzyl-oxycarbonyl, tert.butyloxycarbonyl etc. amino acids as well. Furthermore, some optically pure azlactones were also prepared. Thus, azlactone formation itself is not a sufficient explanation of racemization; the stability of the cyclic intermediate toward bases must also be taken into consideration.

Activated derivatives of S-alkyl-cysteine suffer base catalyzed racemization even when their amino group is blocked by the benzyloxycarbonyl or other urethane-type protecting group. A simple, but not uncontested, explanation is reversible β-elimination

$$Y-NH-CH(CH_2-S-CH_2C_6H_5)-C(O)-X \;\rightleftharpoons\; Y-NH-C(=CH_2)-C(O)-X + {}^-S-CH_2C_6H_5$$

supported by the isolation of the thiobenzyl ester of N-benzyloxycarbonyl-S-benzyl-DL-cysteine. This indicates that benzylmercaptan, one of the products of β-elimination was present in the reaction mixture

$$C_6H_5CH_2-O-CO-NH-CH(CH_2-S-CH_2C_6H_5)-C(O)-O-C_6H_4-NO_2 \;\rightarrow$$

$$C_6H_5CH_2-O-CO-NH-CH(CH_2-S-CH_2C_6H_5)-CO-S-CH_2C_6H_5 \;+\; {}^-S-CH_2C_6H_5 \;+\; {}^-O-C_6H_4-NO_2$$

Experiments with S^{35} labeled benzylmercaptane, however, showed no incorporation of radioactivity. Also, racemization appears to be faster than deuterium exchange at the α-carbon atom. Thus racemization via β-elimination might occur only at elevated temperature, while other mechanism(s) could be opera-

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Racemization

The same can be said about base-catalyzed racemization of amino acids. A negative charge on the carboxylate hinders further proton abstraction from the α-carbon of amino acids; dianions

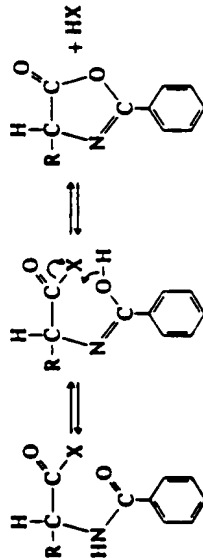$$H_2N-C(R)(H)-C(O)-O^- + OH^- \;\longrightarrow\; H_2N-C(R)-C(O)-O^-$$

are not stable in protic solvents. Therefore, operations involving the amino acids themselves, for instance introduction of the benzyloxycarbonyl group, are carried out in distinctly alkaline solution. In fact, excess alkali prevents the formation of reactive derivatives, such as mixed anhydrides, which might be prone to racemization. In the absence of a free carboxyl group, as in alkyl esters of peptides, base catalyzed racemization does indeed occur during saponification with alkali.
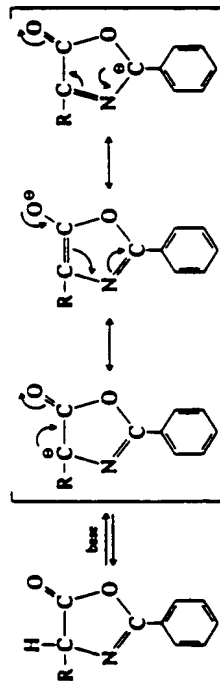
As indicated in the last paragraph, the _activated carboxyl group poses the main problem in the preparation of optically homogeneous peptides._ The electron-withdrawing effect of the activating group (X) extends to the α-carbon atom, the chiral center, and facilitates the abstraction of the hydrogen atom in the form of a proton

$$Y-NH-C(R)(H)-C(O)-X \;\underset{H^+}{\overset{base}{\rightleftharpoons}}\; Y-NH-C(R)-C(O)-X$$

This kind of simple proton abstraction is, however, not the sole, and not even the most common, mechanism of racemization. The most frequently involved pathway involves cyclic intermediates, 4,5-dihydro-oxazole-5-ones or _azlactones:_
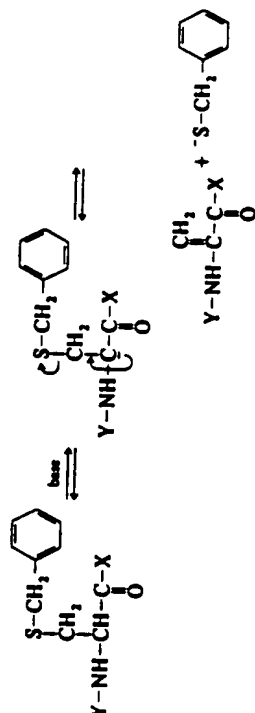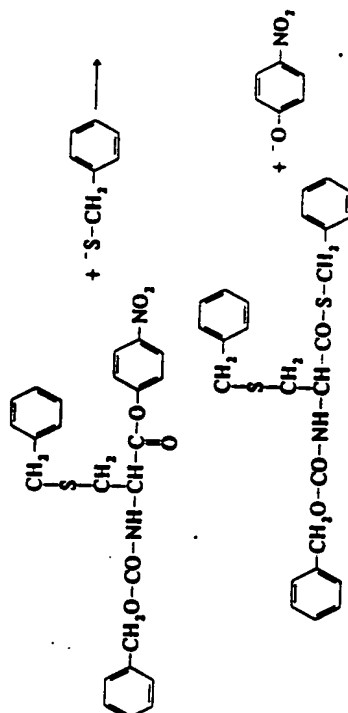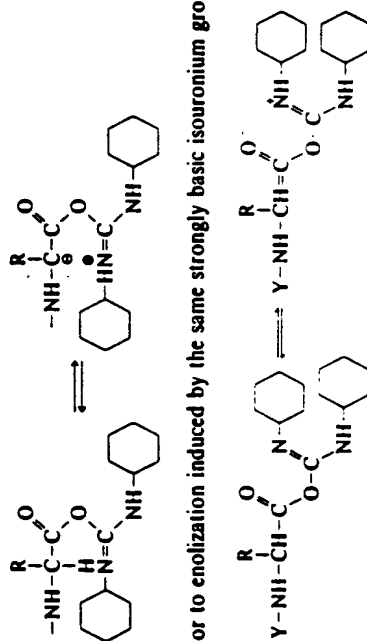
Proton abstraction from the chiral center yields, a resonance stabilized anion

bond fission, however, is a function of the amino acid cleaved from the N-terminus. Very slow rates can be achieved in the hydrolysis of glycyl and prolyl peptides with leucineaminopeptidase (a misnomer, since it is not specific for leucine), fewer difficulties are encountered with aminopeptidase M. Fast and complete hydrolysis of proline containing peptides requires the use of prolidases. A mixture of two enzymes, e.g. aminopeptidase M and prolidase can be quite efficient. Similar results are obtained with carboxypeptidases that provide stepwise removal of single amino acids starting with the C-terminal residue. Carboxypeptidase A has reduced catalytic effect when basic amino acids occupy the terminal position while carboxypeptidase B is most efficient just in this case. The yeast enzyme, carboxypeptidase Y is a more general catalyst.

A considerable number of biologically active peptides end with carboxamide rather than with a free carboxyl group. These peptide amides are, of course, no substrates for carboxypeptidase. An analogous problem exists in peptides which carry an acyl group such as the acetyl group at their N-terminus and, accordingly, can not be degraded with aminopeptidases. If both exopeptidase enzyme types fail, one can resort to a preliminary fragmentation of the chain with endopeptidases, such as trypsin. The latter is very specific for basic amino acids and catalyzes hydrolytic cleavage of the bond between arginine and the next residue and of the bond that follows lysine. The tryptic fragments then are suitable for further enzymatic degradation with exopeptidases, particularly with carboxypeptidase B. Chymotrypsin is similarly useful, but its specificity is somewhat less pronounced: in addition to the bond which follows an aromatic amino acid, the bond after a leucine residue is also cleaved, albeit at a slow rate. In hydrolyzates obtained with proteolytic enzymes only amino acids should be present; uncleaved peptides reveal the presence of a D-residue.

Acid catalyzed hydrolysis followed by the identification of D-amino acids in the hydrolysate is equally useful. To make this possible the amino acids in the mixture are acylated with an enantiomerically pure amino acid, for instance with the N-carboxyanhydride of L-leucine. In the resulting mixture of dipeptides any racemized residue is revealed by the formation of *two* dipeptides that are diastereoisomers of each other, for instance L-leucyl-L-phenylalanine and L-leucyl-D-phenylalanine. Since these are compounds with different physical properties they are separable and appear as a doublet on recordings of an amino acid analyzer. In recent years the conversion to diastereoisomers became unnecessary because the availability of chiral supports now permits separation of enantiomers by high pressure liquid chromatography (HPLC) and also by thin layer chromatography on plates covered with a chiral layer.

C. Racemization Studies in Model Systems

Racemization during the synthesis of peptides is a complex problem. The diversity of possible courses followed in the process is compounded by the

tive under the conditions usually maintained during coupling. Among the various hypotheses that were put forward the partial acceptance of the negative charge of the carbanion intermediate by the d-orbitals of the sulfur atom is somewhat contradicted by the racemization of O-benzyl-serine derivatives.

The mechanisms described in the preceding paragraphs are the ones generally proposed for the explanation of racemization, but it is far from certain that other pathways are not involved. For instance it seems to be possible that the repeatedly observed loss of chiral integrity of the activated residue in coupling of peptides with the aid of dicyclohexylcarbodiimide is due to *intramolecular* proton abstraction by the basic center in the reactive O-acylisourea intermediate

or to enolization induced by the same strongly basic isouronium group:

Thus the acidic character of additives such as 1-hydroxybenzotriazole contributes to their ability to prevent racemization in coupling with carbodiimides.

B. Detection of Racemization

Loss of chiral homogeneity is an always present risk in peptide synthesis and there is an obvious need for methods that can reveal the presence of undesired diastereoisomers in the intermediates and particularly in the final product of the chain-building procedure. With carefully developed chromatographic systems it is often possible to separate fairly long peptide chains which are different from each other only with respect to the configuration of a single amino acid residue. There are however several methods available for this kind of scrutiny that can be applied without a special study of the particular product in question. Such general methods require hydrolysis of the peptide either with *acid* or with the aid of *proteolytic enzymes*. The specificity of these enzymes is the major advantage of the enzymatic approach: no hydrolysis occurs between a D-residue and the next amino acid in the sequence. Therefore complete hydrolysis will take place only in peptides that contain no D residues. The rate of peptide
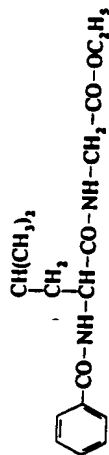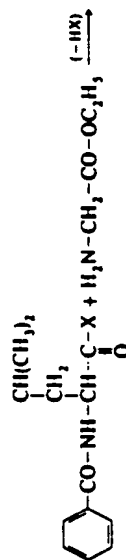
individuality of the amino acids. This was already shown in the example of S-alkyl-cysteine residues, which lose chiral purity by a special mechanism even if protected by a urethane-type protecting group that prevents racemization in other acylamino acids. The opposite end of the scale is represented by proline, which, at least under the commonly applied conditions of peptide synthesis, resists racemization. This was conventionally explained by the circumstance that proline is a secondary amine and, therefore, in its N-acyl derivatives lacks the hydrogen atom which participates in the formation of azlactones (cf. section A in this chapter). The experience, however, gained with readily racemized N-acyl derivatives of N-methylamino acids contradicts this assumption. It appears more likely that the rigidity of the cyclic side chain of proline excludes certain transition states that are integral parts of the racemization process.

Various side chains affect the extent of racemization in different ways. Thus, the benzyl side chain in phenylalanine contributes to the stabilization of a carbanion and can thereby facilitate proton abstraction from the α-carbon atom. This effect is much more pronounced in phenylglycine (which is not a protein constituent but occurs in microbial peptides) because its chiral carbon atom is benzylic:
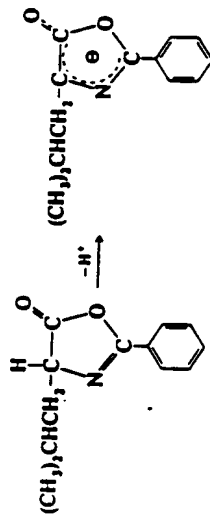
The aliphatic side chains in alanine and leucine have no major influence but branching at the β-carbon atom in valine and isoleucine can enhance racemization because the combination of electron release and steric hindrance results in reduced coupling rates. The ensuing increase in the life-time of the reactive intermediate provides an extended opportunity for proton abstraction by base. It is obvious from these examples that the effect of individual side chains, the influence of various methods of coupling and the conditions of the peptide bond forming reaction (solvents, concentration, temperature, additives) must be studied in well designed experiments. Several model systems have been proposed for this purpose.

The first model (Williams and Young 1963) was based on coupling of benzoyl-L-leucine to glycine ethyl ester. The specific rotation of the crude product was used

to establish enantiomeric purity. This simple system soon became popular and provided valuable information. Some shortcomings of the method must also be taken into consideration. The benzoyl group is not the best representative of blocking groups or of the part of the peptide chain that acylates the activated residue: it is more conducive to azlactone formation and might contribute to the stability of the anion generated from the azlactone by proton abstraction:
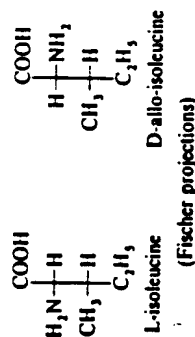
Therefore the Young-test might lead to somewhat exaggerated estimates of racemization. This distortion is counterbalanced by the relative resistance of leucine to racemization, but an additional problem is created by the necessity of isolating the crude benzoyl-leucyl-glycine ethyl ester in excellent yield. If less than near quantitative yield is achieved in coupling or in recovery of the product, there remains the possibility that the unaccounted portion contains a not insignificant amount of the D-isomer.

A frequently used early model (Anderson and Callahan 1958) is based on the coupling of benzyloxycarbonyl-glycyl-L-phenylalanine to glycine ethyl ester. Since the phenylalanine residue is acylated by glycine and not by the benzyloxycarbonyl group, it is not protected against racemization. Accordingly, reactions which cause loss of chiral purity produce in addition to Z-Gly-L-Phe-Gly-OEt also its enantiomer Z-Gly-D-Phe-Gly-OEt. The extent of racemization is easily established from the amount of the racemate because it separates from dilute ethanol. However, the results of this test are reliable only if the peptide bond forming reaction proceeds with excellent yield. The presence of byproducts can grossly interfere with crystallization and no racemate might separate although the D-isomer has been produced in considerable amount. In general: it is risky to rely on negative evidence, the *lack* of separation of the racemate.

Several later model systems were designed with the thought of separating products that are not enantiomers but diastereoisomers of each other. For instance in the coupling of acetyl-L-isoleucine to glycine ethyl ester racemization will yield acetyl-D-alloisoleucyl-glycine ethyl ester, because inversion at the

α-carbon atom leads to a D-amino acid while chirality at the second chiral center, the β-carbon atom is unaffected

COOH		COOH	
H₂N—	—H	H—	—NH₂
CH₃—	—H	CH₃—	—H
	C₂H₅		C₂H₅
L-isoleucine		D-allo-isoleucine	

(Fischer projections)

and hence, an alloisoleucine derivative is obtained. Complete hydrolysis (e.g. with 6 N HCl at 110° for 16 hrs) cleaves the amide and ester bonds and the hydrolysate can be applied to the column of an amino acid analyzer. In the well established Stein-Moore, method of amino acid analysis, isoleucine and alloisoleucine appear as well separated peaks and their ratio provides the information sought about the extent of racemization. The method does not require separation of the two peptides and therefore the results are not modified by imperfections in the operations of recovery. In more general versions of the same idea, diastereomeric tripeptides are produced, deblocked and compared with the help of the amino acid analyzer as such, that is without hydrolysis. For instance benzyloxycarbonyl-glycyl-glycyl-L-alanine is coupled to L-leucine benzyl ester and after hydrogenation the mixture containing glycyl-L-alanyl-L-leucine and glycyl-D-alanyl-L-leucine is applied to the column of the instrument. By replacing L-alanine with other L-amino acids important information can be gained about the sensitivity of various amino acids to a certain coupling method or the conditions of coupling.

Volatile peptide derivatives, for instance trifluoroacetyl-L-valyl-L-valine methyl ester or benzyloxycarbonyl-L-leucyl-L-phenylalanyl-L-valine tert.butyl ester can be separated from their diastereoisomers that contain a D-residue by vapor phase chromatography. Also, through the examination of nmr spectra of relatively simple peptides the extent of racemization that occured during their preparation can be determined without separation of the diastereoisomers, because the difference in the chemical shifts of some selected resonances is sufficient for integration. Thus the areas under the well separated peaks of the alanine methyl protons in acetyl-L-phenylalanyl-L-alanine methyl ester and in acetyl-D-phenylalanyl-L-alanine methyl ester can be integrated and the values used to determine the extent of racemization of the phenylalanine residue during coupling.

These are only selected examples of the numerous model systems proposed for the study of racemization, yet, even in such a brief treatment an approach based on enantio-selective enzymes should be mentioned. Coupling of benzyloxycarbonyl-L-alanyl-D-alanine to L-alanyl-L-alanine benzyl ester yields a blocked intermediate from which on catalytic hydrogenation the free L-Ala-D-Ala-L-Ala-L-Ala is obtained. This compound is completely resistant to hydrolysis catalyzed by aminopeptidases, because the first bond to be cleaved links the N-terminal residue to a D-amino acid. If however, racemization took place during coupling, this changed the activated residue, D-amino acid to L-alanine and after deblocking the tetrapeptide L-Ala-L-Ala-L-Ala-L-Ala is obtained. The latter is completely digestible with aminopeptidases. The liberated alanine is determined and it is a rather exact measure of racemization because for each residue inverted four molecules of alanine are found in the analysis.

At this point a comment has to be added concerning the degree of racemization established with the help of model systems. Usually the amount of the undesired diastereoisomer is considered to represent the extent of racemization. While this might be acceptable for the purpose of comparisons, one has to keep in mind that from the achiral intermediate of the process the two isomers formed in equal amounts. Thus the number of molecules involved is twice the number of the undesired diastereoisomers formed.
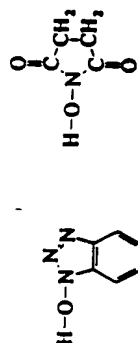
D. Prevention of Racemization

Since racemization during coupling is a base catalyzed process it is reasonable to assume that the nature of the base is not without influence on its outcome. Steric hindrance in some tertiary amines can weaken their ability to approach the chiral center in reactive intermediates. Diisopropylethylamine caused less racemization than triethylamine in the coupling of S-benzyl-L-cysteine derivatives, but it was without significant beneficial effect in reactions involving other amino acids. Perhaps in azlactones, the commonly implicated intermediates of racemization, the chiral carbon atom is well exposed and therefore the bulky groups in the tertiary amine can not interfere with proton abstraction. Also, some differences were found in couplings via mixed anhydrides between the previously preferred base triethylamine and tertiary amines such as N-ethylpiperidine or N-ethylmorpholine, the latter being less conducive to racemization. The principal lesson to be learned is, however, to omit, when possible tertiary amines from the coupling mixture. The often applied approach, addition of a tertiary amine to a salt of the amine component, is certainly inferior to the use of the amine component as such, that is the free amine. Several studies demonstrated that very little if any racemization takes place if this simple principle is followed.

Tertiary amines are added to the reaction mixture also when mixed anhydrides are generated:

$$R-COOH + NR'_3 \longrightarrow R-COO^- \cdot H^+NR'_3$$

$$R-COO^- \cdot H^+NR'_3 + R''-\overset{O}{\overset{\|}{C}}-Cl \longrightarrow R-\overset{O}{\overset{\|}{C}}-O-\overset{O}{\overset{\|}{C}}-R'' + R'_3NH^+ \cdot Cl^-$$
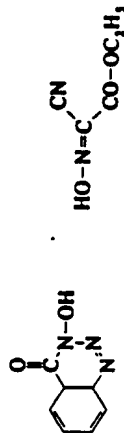
amino group

of the amine component and therefore they do not interfere with its acylation, but their acidity is sufficient to provide competition in abstraction of the proton from the carbon atom of activated intermediates. The significance of these additives is based however, not merely on their acidic character: many other weak acids perform poorly in the role of racemization suppressing agents. Both additives are related to hydroxylamine and function as powerful auxiliary nucleophiles. They react with overactivated intermediates, such as the O-acylisourea in carbodiimide mediated couplings,
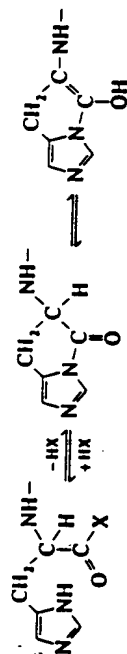

reducing thereby the lifetime of the racemization prone species. The active esters produced in these reactions have higher chiral stability. In their reaction with the amine-component the additive is regenerated and assures a continued beneficial effect. Strangely, the highly efficient additives 3-hydroxy-3,4-dihydro-1,2,3-benzotriazine-4-one (König and Geiger 1970b) and 2-hydroximinocyanoacetic acid ethyl ester (Itoh 1973) have not been widely used so far although their effect on the prevention of racemization exceeds that of the popular 1-hydroxybenzotriazole.
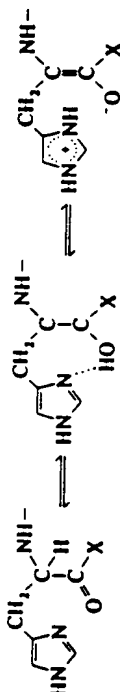

The rather general measure that can be taken against side reactions, the use of both the carboxyl-component and the amine-component in high concentration, is applicable for the suppression of racemization as well. However, poor solubility of intermediates, sometimes even in dimethylformamide, presents a formidable obstacle compounded by the high molecular weight of some blocked peptides. Thus a high molar concentration of both components is often

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...erstandably, production of the same mixed anhydrides is accompanied by ...ess racemization if it is carried out with the help of 1-ethyloxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), because no addition of tertiary base is required and the basicity of the quinoline formed in the reaction is negligible. It is more difficult to counteract the effect of an intramolecular basic center, even if weak, such as the imidazole nucleus in the histidine side chain. While the here shown cyclization and enolization

do not eliminate the ability of the activated species to acylate the amine component (acylimidazoles are good acylating agents) the chiral integrity of the histidine residue may certainly suffer in the process. Racemization via enolization might occur without cyclization as well, particularly because the enol can be stabilized in enolate form:

Hence it appears advantageous to further reduce the basic character of the imidazole by blocking, preferably at the π-nitrogen atom.

It is not surprising that a process involving proton abstraction is influenced by the polarity of the solvent. Base catalyzed racemization of active esters is fast in polar solvents such as dimethylformamide and slow in non-polar media, for instance in toluene. It is rather unfortunate that such non-polar solvents are more often than not impractical in peptide synthesis. The poor solubility of most blocked intermediates in the commonly used organic solvents severely limits their use and in the preparation of larger peptides indeed dimethylformamide is most frequently applied. The problem of solubility is less serious in solid phase peptide synthesis (cf. Chapter X), where no real solvent is needed but merely a medium in which the polymeric support properly swells. This function is fulfilled by dichloromethane; its effect on racemization lies between the extremes mentioned.

Proton abstraction from the chiral carbon atom can be suppressed by the addition of weakly acidic materials to the reaction mixture. Of the numerous additives tested 1-hydroxybenzotriazole (König and Geiger 1970a) and N-hydroxysuccinimide (Weygand et al. 1966) are routinely used in the practical execution of coupling. These compounds are not acidic enough to protonate the

IX. Design of Schemes for Peptide Synthesis

In the strategical planning that must precede the synthesis of a larger peptide, racemization is one of the most important considerations. Therefore, it seems to be appropriate to discuss the various schemes of synthesis at this point. Due to the individuality of amino acid residues and to variations in the properties of blocked intermediates, it appears impractical to propose a general scheme (strategy) that would be applicable for any peptide. Peptide synthesis should be based on retrosynthetic analysis, starting with identification of the problems inherent in the sequence of the target compound.

In principle three approaches are possible: A. condensation of peptide segments; B. stepwise chain-building starting with the N-terminal residue; and C. stepwise chain building starting at the C-terminus. We will attempt to evaluate these alternatives, but with some reservation: there is no consensus among peptide chemists in this area.

A. Segment Condensation

In the earliest days of practical peptide synthesis, in the preparation of the nonapeptide oxytocin or the octapeptide angiotensin, segment condensation appeared to be the obvious strategy. Reduction of a major task to smaller problems, a Cartesian approach, is clearly attractive. Equally important is, however, the possibility of dividing the effort among members of a team. Preparation of the individual segments, often dipeptides, could be entrusted to less experienced coworkers while the arduous task of segment condensation needs an adept in peptide chemistry. A similar distribution of responsibilities is not feasible in stepwise chain lengthening. These considerations must have guided the investigators who undertook the synthesis of biologically active peptides in the nineteen fifties. The retrosynthetic scheme for the synthesis of the octapeptide angiotensin is shown here as an example:

Asp—Arg—Val—Tyr—Ile—His—Pro—Phe
⇩
Asp—Arg—Val—Tyr+Ile—His—Pro—Phe
⇩
Asp—Arg+Val—Tyr   Ile—His+Pro—Phe
⇩   ⇩   ⇩
Asp+Arg   Val+Tyr   Ile+His   Pro+Phe

---

tainable. On the other hand, if the carboxyl component is not a peptide derivative but rather the blocked and activated form of a single amino acid, it can be used in excess. This excess can be adjusted to provide for a concentration which remains sufficiently high (for instance more than 0.1 molar) throughout the coupling reaction. The relative simplicity of the blocked *and* activated amino acid derivatives and their availability from commercial sources render this sacrifice usually acceptable. Over and above the possibility of performing coupling reactions according to the *principle of excess*, addition of single amino acid residues has a further important benefit in the conservation of chiral purity: the most commonly used amine-protecting groups, the benzyloxycarbonyl and the tert.butyloxycarbonyl group, efficiently prevent racemization in most cases. As mentioned before, this is a common feature of urethane-type blocking groups and applies for the acid-stable, base-sensitive 9-fluorenyl-methyloxycarbonyl (Fmoc) group as well.

In the preceding discussion we have dealt only with racemization during peptide bond formation. Loss of chiral purity can occur, however, also during certain processes of deprotection. Hydrogenolysis is quite innocuous in this respect and acidolysis is harmful only if it is carried out under drastic conditions, such as elevated temperature. Saponification of esters with alkali can cause measurable racemization. This must be kept at a minimum by carrying out the reaction at ice-bath temperature, preferably at constant p_H. Large excess of alkali certainly must be avoided. The presence of Cu^{++} ions prevents racemization in alkaline hydrolysis (and probably also in many instances of coupling), but complete removal of the metal ions from the complex is not always straightforward.

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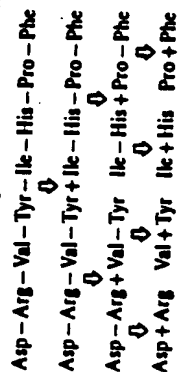   G. T., Williams, M. W.: ibid. p. 3902

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Miklos Bodanszky

Principles
of Peptide Synthesis

Second, Revised Edition

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Preface to the Second Edition

The attempt to render PRINCIPLES OF PEPTIDE SYNTHESIS somewhat resistant to the passing of time could, of course, be only partially successful. In the decade that has elapsed since the completion of the manuscript, the discovery of a long series of biologically active peptides together with the major application of peptide hormones, such as calcitonin, the blood-pressure-lo   ng enzyme inhibitor, the pseudopeptide captopril, in medicine, and the large-scale production of the sweetener, aspartame, have given new impetus to peptide chemistry. A considerably widening of interest in peptide synthesis, both in academia and in industry, ensued and numerous novel methods appeared in the literature. It seemed timely to update the original version of PRINCIPLES OF PEPTIDE SYNTHESIS.

Preparation of this Second Edition provided a welcome opportunity for revising the text. This revision went beyond the correction of printer's errors and other mistakes. A more substantial modification of the first edition was prompted by a thorough critique by Professor G.T. Young of Oxford University. I considered his recommendations carefully and adopted most of them. Some changes in the evaluation of methods have also been made. For instance I reexamined the principle of coupling reagents and introduced the concept of "true coupling reagent".

Only a part of the new procedures could be fitted into the appropriate chapters of the first edition, hence most of th   aterial published between 1982 and 1992 was assembled in the c....luding Chapter VIII. This separation of old and new served not merely convenience but also allowed me to attempt an assessment of new ideas and to discern novel trends.

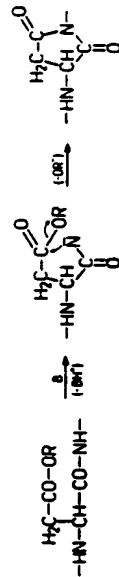Princeton, New Jersey, 1993   MIKLOS BODANSZKY

In esters and in various activated derivatives of acylamino acids, no such obstacle exists against proton abstraction. In fact the electron withdrawing forces present in the activating group "X" enhance the activity of the α-hydrogen and facilitate its abstraction:

$$-HN-\underset{H}{\overset{R}{C}}-CO-X \underset{(-BH^+)}{\overset{B}{\rightleftharpoons}} -HN-\overset{R}{C}-CO-X$$

An obvious consequence of carbanion formation is the partial or total loss of chiral purity. Proton abstraction might be reversible and the equilibrium of the reaction might lie far to the left: gradually more and more molecules will pass through a carbanion stage and suffer irreparable racemization. Therefore, the risk of racemization is inherent in peptide synthesis and in order to avoid it, it must be carefully considered. There are, however, side reactions in which proton abstraction occurs not at the α-carbon atom but at the amide nitrogen of an acylamino acid. The additional

$$-\overset{O}{\underset{}{C}}-\underset{H}{\overset{}{N}}-CHR-CO- \underset{(-BH^+)}{\overset{B}{\rightleftharpoons}} -\overset{O}{\underset{}{C}}-\overset{}{N}-CHR-CO-$$

unshared pair of electrons on the nitrogen atom renders the latter, in spite of the presence of the carbonyl substituent, a good nucleophile. Thus, it can participate in numerous side reactions, particularly in intramolecular attacks resulting in cyclizations. For instance, the formation of succinimide derivatives is usually preceded by proton abstraction from the amide nitrogen of an aspartyl amino acid residue:

[reaction scheme]

Analogous cyclization reactions and O-acylations initiated by proton abstraction will be discussed in separate sections.
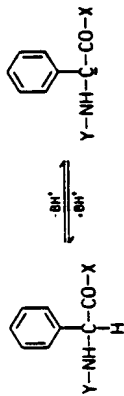
1.1 Racemization

1.1.1 Mechanisms of Racemization

Understanding the mechanisms of racemization seems to be necessary for its prevention. Accordingly, a considerable amount of experimental work has been carried out in this area, and was skillfully rendered in a review article by Kemp [4]. At this place we confine the discussion to the

principal processes of base catalyzed racemization of activated acylamino acids. Three distinct pathways can be recognized:
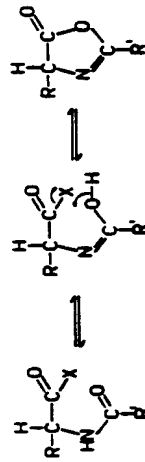
a) direct abstraction of the α-proton,
b) racemization via reversible β-elimination and
c) racemization through azlactones [5(4H)-oxazolones].

The simple proton abstraction mechanism might be a contributor in several processes but it is the dominant pathway only in very special cases such as the rapid racemization of derivatives of phenylglycine, an amino acid which is not a constituent of proteins although it occurs in microbial peptides:

$$Y-NH-\underset{H}{\overset{}{C}}-CO-X \underset{-BH^+}{\overset{-BH^+}{\rightleftharpoons}} Y-NH-\overset{}{C}-CO-X$$

(where Y is a protecting group and X an activating group). The conspicuous racemization of active esters of N-benzyloxycarbonyl-S-benzyl-L-cysteine [5] was usually explained by the reversible, base-induced elimination of benzylmercaptane and this explanation was supported by the fortuitous isolation of N-benzyloxycarbonyl-S-benzyl-DL-cysteine thiobenzylester from a solution of the p-nitrophenyl ester containing triethylamine. Subsequent studies, carried out e.g. with radioactively labelled benzyl-mercaptane [6], demonstrated that racemization of reactive cysteine derivatives can proceed without the elimination of the thiol. Further examination of the problem led to a proposal [7] in which a direct interaction between the chiral carbon-atom and the sulfur atom, involving the d-orbitals of the latter, is invoked. The problem, however, is further complicated by the often observed racemization of reactive derivatives of O-benzyl-serine (in which clearly no d-orbitals are present) during coupling. Thus the d-orbitals of the sulfur atom might contribute to but cannot be solely responsible for the racemization of S-alkyl-cysteine. A rationale, applicable both for cysteine and for serine derivatives, is enol-stabilization by intramolecular hydrogen bonds, with the sulfur or the oxygen atom, respectively, as bridgeheads. The ready racemization noted in reactive derivatives of β-cyano-alanine [8] can be explained simply by the strong electron-withdrawing effect of the cyano group.

The best studied and probably most important mechanism of racemization involves the formation of azlactones [9]:

[reaction scheme]

The explanation for the tendency to racemization of azlactones lies in the case by which the acidic proton can be abstracted by bases from the chiral center due to resonance stabilization of the carbanion generated in the process:

Azlactones are good acylating agents and could be useful for the activation of the carboxyl component. Yet, delocalization of the negative charge in the deprotonated intermediate provides them with sufficient lifetime to endanger the chiral purity of the product. The formation of an azlactone could be demonstrat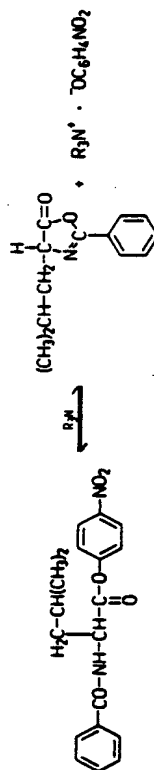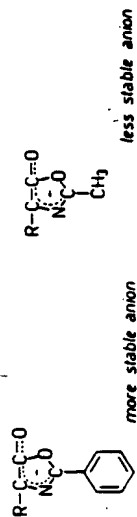ed [10] by its characteristic carbonyl frequency (1832 cm^{-1}) when benzoyl-L-leucine p-nitrophenyl ester was exposed to the action of tertiary amines
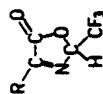

and equally convincing evidence incriminating the azlactone intermediate was found in the production of partially racemized benzoyl-leucyl-glycine ethyl ester when the reaction was completed with acylation of glycine ethyl ester. Characteristically, the unreacted portion of benzoyl-L-leucine p-nitrophenyl ester was recovered enantiomerically pure. Racemization through azlactone intermediates is influenced by several factors such as the nature of the amino acid involved, the solvent used in the reaction or the presence (or absence) of tertiary amines. The acyl group on the amine nitrogen, however, plays a decisive role in the conservation or loss of chiral purity. For instance, under identical conditions, benzoylamino acids are more extensively racemized than acetylamino acids [11]. Such differences seem to be related to the electronic forces operating in the acyl group. Beyond the formation of azlactones the N-acyl substituents of the oxazolinone can also affect the acidity of the hydrogen atom on the chiral center. Expressed in another way; the stability of the anion produced in proton abstraction by bases is enhanced by electron withdrawing effects in the acyl group:

more stable anion   *less stable anion*

It is probably not so much the formation of azlactones that is of primary importance in determining the rate of racemization but rather the electronic effects of the substituents of the oxazolinone, including those in the N-acyl group. Azlactones can be obtained in optically active form [12], and if immediately trapped by good nucleophiles [13], they can yield optically active products.

The influence of the N-acyl group on the stability of the anion generated through proton abstraction from the oxazolinone can range from extreme stabilization found in the formyl and trifluoroacetyl groups to pronounced destabilization shown by the benzyloxycarbonyl, tert-butyloxycarbonyl and other alkoxycarbonyl groups. In fact, trifluoroacetylamino acids yield an isomer [14] of the more common azlactones, an isomer in which the α-carbon atom is not chiral:

Until recently it was generally assumed that benzyloxycarbonylamino acids and, in general, amino acids protected by a urethane-type blocking group do not produce azlactones and hence are resistant to racemization during activation and coupling. Isolation [15] of optically pure oxazol-(4H)-ones, e.g. from the reaction of tert-butyloxycarbonyl-L-valine with water soluble carbodiimides contradicts such assumptions and suggests that the beneficial effect of urethane type protecting groups rests on the electron release provided by them and on the ensuing destabilization of the anion which could form by proton abstraction:

The chiral stability of proline derivatives was usually explained by the absence of an amide hydrogen in the N-acyl derivatives of this secondary amine. It appeared plausible that without such an amide hydrogen no azlactone should form. This explanation, however, ignores the possible

formation of protonated azlactones (oxazolonium salts). It was completely refuted by the ready racemization of N-methylamino acids [16] during activation and coupling. Thus, the chiral stability of proline is due to its rigid geometry rather than the fact that it is a secondary amine. Under certain conditions, e.g. in diketopiperazines, proline is readily racemized.

The role of bases in at least some of the racemization processes is beyond doubt. For instance, time and again the advantage of free amines over a mixture of amine salts with tertiary bases was noted. Less attention has been paid so far to the possibility of *intramolecular* base catalysis, although in several coupling methods the reactive intermediate contains a basic center and the latter could abstract the hydrogen from the chiral carbon atom. Since O-alkyl isoureas have pronounced basic character, it may not be farfetched to assume intramolecular proton abstraction by a basic nitrogen atom in the O-acyl-isourea intermediates of carbodiimide mediated coupling reactions. For instance hydrogen bond stabilized enols might play a role in such processes

which would then be analogous to the effect of excess acetic anhydride on optically active amino acids. Here racemization probably proceeds through enolization of mixed anhydrides:

1.1.2   Models for the Study of Racemization

Numerous model systems have been proposed for the study of racemization. These systems are used to evaluate the effect of solvents, presence or absence of bases, temperature and other variables and last, but not least, the ability of different coupling methods to produce peptides without loss of chiral purity. The earliest suggestions came from Young's laboratory [17, 18] and involve the coupling of acetyl or benzoyl-L-leucine to glycine ethyl ester, followed by the examination of the optical rotation of the crude product. The results can be further refined by fractional crystallization
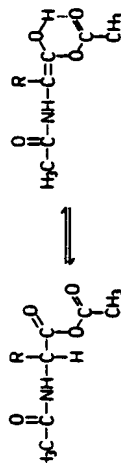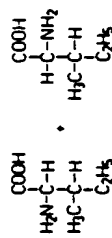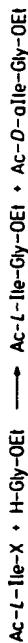
and analysis of the fractions by weight, optical rotation and melting point. The benzoyl group enhances the tendency for racemization, hence activation and coupling of benzoyl-L-leucine is a very sensitive racemization test.

A simple, and therefore frequently applied, model experiment was designed by Anderson and Callahan [19]. It involves the coupling of benzyloxycarbonylglycyl-L-phenylalanine to glycine ethyl ester. If racemization occurs in the process the product contains benzyloxycarbonylglycyl-DL-phenylalanyl-glycine ethyl ester, which is fairly insoluble in aqueous ethanol and can thus be separated and weighed. A word of caution is indicated here. This simple and useful method is reliable only if no by-products, other than the racemate, are formed in significant amount in the coupling reaction. Otherwise crystallization of the racemate might be impeded by the impurities and from the lack of crystallization the wrong conclusion, that there was no racemization, can be drawn. In principle, models should be so designed that the products of the test-experiment are not racemates but diastereoisomers and the conclusions are not based on negative evidence.

A more reliable, albeit also more time consuming, experiment is based on the coupling of benzyloxycarbonylglycyl-L-alanine to L-phenylalanyl-glycine ethyl ester (the "Kenner model") [20]. The diastereoisomers formed in the reaction are separated by countercurrent distribution. Somewhat less laborious are the methods introduced by Weygand and his associates [14, 21, 22], who condensed trifluoroacetyl-L-valine with L-valine methyl ester, or benzyloxycarbonyl-L-leucyl-L-phenylalanine with L-valine *tert*-butyl ester or trifluoroacetyl-L-prolyl-L-valine with L-proline methyl ester. The reaction products are examined with the help of vapor phase chromatography for the presence of diastereoisomers formed by racemization.

The test systems discussed so far are based on differences with respect to solubility or partition coefficient between diastereoisomers (or in the Anderson-Callahan test, between the racemate and the enantiomerically pure peptide derivative). An experimentally simple realization of the same principle is the examination of the products of model reactions by paper chromatography or thin layer chromatography [23]. Improvements in the realiability of the tests are also possible, e.g. the Young test can be perfected by the chromatographic separation of the products [24]. A more substantial simplification is, however, the use of the ubiquitous amino acid analyzer for the separation and quantative determination of the diasterosimers generated in the racemization tests. For instance coupling of acetyl-L-isoleucine [25] to glycine ethyl ester yields, in addition to the desired acetyl-L-isoleucylglycine ester, also acetyl-D-*allo*isoleucylglycine ethyl ester, if racemization occured in the reaction. Since alloisoleucine and isoleucine are routinely separated by the Spackman-Stein-Moore method [26], it is sufficient to

Ac–L–Ile–X + H–Gly–OEt ⟶ Ac–L–Ile–Gly–OEt + Ac–D–aIle–Gly–OEt

H/HOH → H₂N–CH₂COOH +

COOH
|
H₂N–C–H
|
H₃C–C–H
|
C₂H₅

,

COOH
|
H–C–NH₂
|
H₃C–C–H
|
C₂H₅

hydrolyze a small sample of the reaction mixture and to apply the hydro-lysate to the analyzer. The main advantage of this model experiment is that no isolation of products is needed. This means a certain saving of time and effort, but more importantly the examination of the *crude* material assures that no distortion takes place in the isolation or separation of the products, thus no isomer is left in mother liquors, etc. The acetyl group has no major effect on the racemization of the amino acid to which it is attached, thus in this respect it can represent a peptide chain. This model can be applied for the study of the effect of coupling methods, solvents, tertiary amines added and also of the influence of the amino component, since glycine ethyl ester can be replaced by other nucleophiles. Yet, a certain limitation is caused, by the choice of isoleucine as the activated residue. It is a hindered amino acid and might suffer more loss in chiral purity than other less hindered residues which do not reduce the rate of the desired reaction and therefore allow less time for unimolecular processes such as racemization.

The same principle, separation of diastereoisomers on the amino acid analyzer, appears also in the "Izumiya test" [27, 28] in which a benzyloxy-carbonylglycyl amino acid is coupled to an optically active amino acid benzyl ester and the products examined after deprotection by hydrogena-tion. This model system allows variations with respect to the amino acid residue which is exposed to racemizing conditions. Thus, instead of Z-Gly-L-Ala one can couple Z-Gly-L-Phe, etc. to L-Leu-OBzl and the nucleophile can also be so selected that detection of the diastereoisomers causes no difficulty. The contributions of Benoiton and his associates [29, 30], who used Nᵋ-benzyloxycarbonyl-L-lysine benzyl ester for amino component, lie in the same direction. The degree of racemization can be estimated, without deprotection and separation, through the examination of the nmr spectra of the coupling products. The model compounds acetyl-L-alanyl-L-phenylalanine methyl ester and acetyl-L-phenylalanyl-L-alanine methyl ester [31] allow the determination of the D-amino acid containing isomers by integration of the areas of the methyl protons of alanine while coupling of benzoylamino acids to Nᵋ-benzyloxycarbonyl-L-lysine methyl ester [32] permits a similar assessment of racemization through the examination of the methyl protons of the methyl ester group. In an interesting proposal [33] coupling of tert-butyloxycarbonyl-L-alanyl-L-methionyl-L-leucine to the tert butyl ester of L-leucine is followed by acidolysis and then by a treatment with cyanogen bromide in aqueous

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acetic acid and by determination of the ratio of the two diastereoisomers, L-Leu-L-Leu and D-Leu-L-Leu with the help of the amino acid analyzer:

Boc–Ala–Met–Leu + Leu–OBuᵗ ⟶ Boc–Ala–Met–Leu–Leu–OBuᵗ —CF₃COOH→

H–Ala–Met–Leu–Leu–OH —BrCN→ Ala–Hse + L–Leu–L–Leu + D–Leu–L–Leu

Hse = Homoserine

In a sophisticated and also very sensitive model experiment [34] benzyloxycarbonyl-L-alanyl-D-alanine is activated by the method to be tested and coupled to L-alanyl-L-alanine p-nitrobenzyl ester. The crude product is deblocked by hydrogenation and the mixture of the two isomeric tetrapeptides L-Ala-D-Ala-L-Ala-L-Ala and L-Ala-L-Ala-L-Ala-L-Ala, is exposed to the action of leucine aminopeptidase. The enzyme will catalyze the complete hydrolysis of the all-L peptide, the product of racemization, but leaves the peptide in which the second position is occupied by a residue with D-configuration intact. With respect to sensitivity this method is surpassed by the isotope dilution techniques introduced into peptide chemistry by Kemp and his coworkers [35–37]. Radioactively labeled benzyloxycarbonylglycyl-L-leucine or benzoyl-L-leucine is coupled to glycine ethyl ester followed by dilution with "cold" racemate and fractional crystallization until products with constant count per mg are obtained. This yields reliable information on racemization and allows the detection of very slight racemization which would be left unnoticed in the original versions of the Anderson-Callahan or the Young tests (cf. above).

Some problems, e.g. the base catalyzed racemization of active esters of protected amino acids or peptides can be investigated simply by following the change of optical rotation with time [38]. The effect of solvents, protecting groups, temperature, activating groups, etc. can be studied in this simple manner. With selected model compounds [39] it was possible to determine the scope and limitations of hindered amines in preventing racemization.

1.1.3   Detection of Racemization (Examination of Synthetic Peptides for the Presence of Unwanted Diastereoisomers)

Racemization during the activation and coupling of suitably protected amino acids occurs rarely but cannot be excluded. It is even more likely to occur in the activation and coupling of protected peptides. Therefore, it is desirable and sometimes absolutely necessary to examine the synthetic products for the presence of unwanted diastereoisomers. Such contamin-ants, if they are only minor constituents in the crude synthetic material, might be lost in the isolation process or during purification but can also accompany the principal product through these steps. A simple and

enzymes from snake venoms. The evidence obtained in these oxidative processes should be trusted only if the catalytic effect of the enzyme preparation and the conditions used are shown to be operative in control experiments with mixtures containing both L and D amino acids.

Chromatographic procedures based on columns containing chiral supports [46, 47] can differentiate between D and L amino acids. This principle, perfected by the use of high pressure liquid chromatography, might become the standard control process for the detection of racemization that occurred in the synthesis of a peptide. Reversed phase high pressure chromatography is well suited [48] also for the implementation of the Manning-Moore procedure [40] because well selected columns can completely separate the diastereoisomers formed on acylation of the amino acids in a hydrolysate with an optically pure acylating agent.

1.1.4 Conservation of Chiral Purity

Chiral purity of activated residues is affected by several factors, such as the methods of activation and protection or the nature of the activated amino acid residue. It is influenced also by the solvent used in the reaction, the presence or absence of tertiary amines, and by the basic strength and bulk of the tertiary amine if one had to be added to the coupling mixture and, last but not least, by auxiliary nucleophiles (cf. Chapter II). First and foremost of these factors seemed to be *the method of activation* and thus it received the most attention. The search for "racemization free" coupling methods is still actively pursued although this effort is fraught with an inherent difficulty. Any increase in the activation of the carboxyl group entails an increase in the acidity of the proton on the chiral α-carbon atom and facilitates, thereby, racemization via proton abstraction:

$$-HN-\overset{R}{\underset{H}{C}}-\overset{O}{\overset{\|}{C}}-X + B \;\rightleftharpoons\; -HN-\overset{R}{\underset{}{C}}-\overset{O}{\overset{\|}{C}}-X + BH^{+}$$

It might be more profitable to focus attention on each and every factor influencing racemization, rather than to try to develop perfect coupling methods which will yield chirally pure products under any conditions.

Through decades the strong belief prevailed that the azide method is free from racemization. Only later did we become aware of measurable racemization in azide coupling [21, 49, 50]. Those who observed no racemization in the preparation of peptides via azides (e.g. Ref. [27]) knowingly or intuitively avoided the use of tertiary bases, or at least did not apply tertiary amines in excess [51]. By no means do we suggest that all methods are equal in this respect. The azide method still stands out as

practical approach to the detection of diastereoisomers was devised by Manning and Moore [40]. A sample of the peptide is completely hydrolyzed with constant boiling hydrochloric acid and the mixture of liberated amino acids is acylated with an enantiomerically pure protected and activated amino acid, e.g. with L-leucine N-carboxy-anhydride. The resulting mixture of dipeptides is applied to the column of an automatic amino acid analyzer [26] which can separate dipeptides from their diastereoisomers. Accordingly, if racemization occurred at one or more residues, then, in addition to the peaks corresponding to the expected dipeptides (L-leucyl-L-amino acids) smaller satellite peaks will also appear on the recordings, demonstrating the presence of L-leucyl-D-amino acids in the mixture. The areas under the peaks allow the quantitative determination of the amount of D-amino acids in the synthetic material. There is, of course, an inherent limitation in the examination of chiral integrity of a peptide through its hydrolysis with acids, if the process of hydrolysis itself is not unequivocal in this respect. In acid hydrolysates, most amino acids appear more or less intact, but some, e.g. phenylalanine, suffer minor racemization during hydrolysis, while cystine becomes heavily contaminated with its D-isomer and also with mesocystine. Alkaline hydrolysis is even worse. It causes extensive racemization in several residues. Such details must be taken into consideration in the evaluation of the Manning-Moore analysis. This problem can be eliminated by using proteolytic enzymes for degradation.

The selectivity of proteolytic enzymes also permits their direct application for the study of optical homogeneity [41]. For instance complete digestibility of a sample with leucine amino peptidase [42, 43] provides strong evidence for the absence of D-amino acid containing peptides. A comparison of the ratios of amino acids in hydrolysates obtained on digestion of a synthetic product with proteolytic enzymes with the ratios determined in a routine acid hydrolysate is probably one of the simplest approaches for the study of chiral integrity.
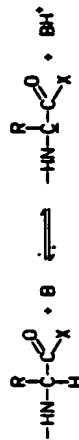
The rates of hydrolysis in degradation with proteolytic enzymes are usually low at bonds following proline and glycine residues. Some aminopeptidases, e.g. aminopeptidase M, are less restrictive in this respect. Proline, a stumbling block in proteolysis, can be set free with the help of specific prolidases [44, 45]. In addition to aminopeptidases, carboxypeptidases A, B and Y, and dipeptidylaminopeptidases can also be adopted for the same purpose. Selective cleavage, e.g. with trypsin at the carboxyl side of arginine and lysine residues, provides useful information if these were the activated amino acids of carboxyl components. In general, the stereospecificity of enzyme catalyzed hydrolysis can serve the study of optical purity in numerous ways. Perhaps less reliable is an alternative approach in which one follows the disappearance of D-amino acids from hydrolysates on treatment with D-aminoacid oxydases (e.g. from kidneys) or the elimination of L-amino acids by oxidation with

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of :
     Gautvik et al.   :

Serial No. 08/340,664   :   Group Art Unit 1812

                            :   Examiner: L. Spector
Filed: November 16, 1994   :

For: PRODUCTION OF HUMAN   :
     PARATHYROID HORMONE FROM :
     MICROORGANISMS   :
_____X

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF KAARE M. GAUTVIK, M.D. PURSUANT TO 37 C.F.R. § 1.132

Sir:

    I, KAARE M. GAUTVIK, declare as follows:

    1. I am a coinventor of the above-captioned application.

    2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as exhibit A.

    3. Throughout the 1980's, and continuing today, I have had a keen interest in a number of medical conditions including osteoporosis. In the early 1980's not much was known regarding this condition. As best exemplified by the *Brewer et al.* patent cited by the Examiner in the Official Action dated September 8, 1995, most of the emphasis at the time was on the N-terminal region, that could bind to certain receptor cells in bones. But the hPTH peptide was not well characterized and certain phenomena could not be explained by this binding. I and others sought to explain these hitherto unexplained phenomena. To test various theories, a good, inexpensive supply of very pure hPTH was needed. At the time, the only way to obtain hPTH was by extraction and isolation, followed by complex purification from human tissue. This was more than just a laborious process. Due to the difficulty in obtaining human tissue where hPTH had not deteriorated, relatively little material could be extracted and isolated at any one time.

Moreover, purification technology had not advanced to the point where suitable purity could be obtained.

4.   Before I could continue my desired research, I first had to find or develop a suitable source of essentially pure hPTH. I felt that if suitable recombinant systems could be developed for producing hPTH, the inherent protein editing mechanism contained within the cell could be harnessed to produce, intact, correctly sequenced, fully active, hPTH. If this was accomplished, purification of the expressed protein could be carried out using the technologies that were prevalent at the time. I therefore sought the skills of my coinventors, and together we developed a source of raw material. The manner in which that was accomplished, and the resulting highly pure peptide, is described my above-captioned patent application.

5.   By the use of recombinant technology as described in the patent application, we have been able to obtain hPTH which is not only of significantly higher purity than anything otherwise available, but also hPTH which was qualitatively superior. The data presented herein describing the attributes of the essentially pure, recombinant hPTH we developed are based on hPTH hormone produced by me or under my direct supervision in the mid to late 1980s. The resultant peptide was purified as described in the application. No other purification steps were employed.

6.   Attached as Exhibits B-E are a number of glossy photographs labeled Glossy 0 through Glossy III. These glossies contain, among other things, photographs of electrophoresis gels run by me or under my direct supervision. Glossy 0 (Exhibit B) corresponds to an electrophoretic gel comparing synthetic hPTH obtained from the chemical supply company, Sigma, to recombinant hPTH obtained from yeast as disclosed in the above-captioned application. This gel was prepared before the filing of my patent application which issued as U.S. Patent No. 5,420,242. Lanes 1 and 4 contain chemical markers. Lane 2 (second from the left) contains recombinant hPTH prepared in accordance with the present invention. The Sigma material was loaded in Lane 3 (third from the left). The symmetrical blurring on either side of the actual hPTH band is the result of overloading the gel. It is significant to note that a single band of material is present in Lane 2 while three distinct bands are found in

Lane 3. This indicates the presence of significant quantities of high molecular weight impurity in the Sigma material.

7. Glossy I (Exhibit C) illustrates the gel of an experiment that was run on November 30, 1989 and shows a comparison screening of different chemically synthesized hPTH peptides from various companies including Peptide (Lanes 3 and 12), Peninsula (Lanes 4 and 13), Sigma (Lanes 5 and 14) and Bachem (Lanes 6, 7, 15 and 16). Two preparations of Bachem material were run (Lot Nos. ZE567 and 734B). Gels were loaded with either 200 nanograms or 800 nanograms of material as indicated, according to the manufacturers instructions. These materials were run against three lanes with molecular markers (Lanes 2, 11 and 20) as well as our recombinant hPTH produced from *E. coli* (Lanes 8 and 17), yeast (Lanes 9 and 18) and QPTH (Lanes 10 and 19). The materials obtained from Peptide and from Peninsula ran as a higher molecular species of much less quantity than indicated by the manufacturer and no correctly sized hPTH could be seen in the Peptide lanes, even when applied at 800 nanograms. The Peninsula material in Lane 4 shows a small indication of correct hPTH, but most of the material exists as a high molecular weight form. The Sigma preparation ran at a correct location but contained much less material than the manufacturer indicated. No material was evident at a loading of 200 nanograms. The two different batches from Bachem show a peptide of correct molecular size, but one of the preparations shows a heavy, and the other a lesser, trailing smear indicating lower molecular weight impurities. Again, the amounts of hTPH contained in the Bachem bands appear to be less than the amounts contained in the bands corresponding to the same loaded amount of recombinant hPTH from yeast and *E. coli*. Each of the three recombinant hPTHs appear as very sharp, fat bands, of equal intensity and much stronger intensity than any of the chemically synthesized preparations. When applied as 200 nanograms, only the recombinant hPTH lanes can be clearly seen. Everyone familiar with gel electrophoresis of protein knows that as little as 100 nanograms is usually sufficient to provide detectable staining. Thus, the absence of staining of 200 nanograms and diffuse bands at 800 nanograms are indicative of a relatively impure peptide.

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8.   Glossy II (Exhibit D) is a different experiment carried out on the same day as Glossy I.   As indicated, two amounts of various materials, 200 nanograms and 600 nanograms, were applied per lane.   The *E. coli* preparation used in this electrophoresis showed two high molecular weight bands on the silver nitrate stained gel (dimeric/polymeric aggregates) (Lanes 24 and 29), while the two yeast hPTH lanes contain very sharp bands in comparison to the Bachem preparation (compare Lanes 22 versus 23 and 27 versus 28, respectively).   The Bachem preparation, shown in Lanes 23 and 28 shows considerable trailing toward degradation products.   The amount of the correct material of the Bachem preparation may best be judged by the electrophoresis of the 200 nanogram sample.   At 200 nanograms of material loaded, very little Bachem material was observed and lower molecular weight species are seen as a trailing area.

9.   Glossy III (Exhibit E) is a blow-up of Glossy II indicating the size of the molecular markers in the first lane on the left.   We have since carried out more recent electrophoresis and the bands appear exactly as they did in 1988 and 1989, indicating that there was no degradation of our preparation since its production in the late 1980's.   The absence of degradation also indicates the substantial purity of the resulting material.   The foregoing clearly indicates the superior purity of the material resulting in accordance with the present invention.   However, it is also my opinion that because the hPTH produced in accordance with the present invention is recombinant material purified as explained in the application, not only is the peptide of better purity, but it is also of a significantly better quality.   The differences are aptly illustrated in some of my prior published works.

10.   For example, attached hereto as Exhibit F is a copy of my paper, "Differences in Binding Affinities of Human PTH(1-84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms," published in the refereed journal, *Peptides*, (1994), 15, 1261-65.   The data reported in this paper involved the analysis of hPTH material produced in accordance with the patent application in the mid to late 1980's.   In fact, no other purification steps were taken, other than those disclosed in the patent application prior to the analyses described in this paper.

11. As will be readily apparent, we ran a number of
in vitro tests to determine not only the purity, but also the
qualities of the recombinant hPTH material prepared in
accordance with the present invention and compared same to the
best available chemically synthesized hPTH from Bachem. The
first test, as illustrated in Fig. 1, shows the inhibition of
radiolabeled [Tyr^{36}] chicken PTHrP (1-36) amide by various
hPTHs. The data represents the mean (± standard deviation) of
at least two independent experiments each performed in
triplicate. As will be self evident from the figure, chemically
synthesized hPTH had a calculated binding affinity, (K_d) of 18nM
(95% confidence interval: 16.1-20.0nM) while recombinant hPTH
(1-84) from both yeast and *E. coli* had a significantly lower
apparent K_d of 9.5nM (95% confidence interval: 8.7-10.4nM).

12. Fig. 2 illustrates the stimulation of cAMP by
different types of hPTH. The recombinant hormones in accordance
with the present invention have the ability to stimulate
intercellular cAMP accumulation with an EC_{50} of about 1.5nM,
(95% confidence interval 1.0-2.2nM). In contrast, the solid
phase synthesized hPTH showed a significantly reduced potency in
stimulating cAMP production with an EC_{50} value of 5.7nM (95%
confidence interval: 3.4-9.6nM). Fig. 2 also illustrates that
the synthetically produced hPTH exhibited a reduced maximal
response. Therefore, no matter how much synthetic hPTH is
administered, it is not possible to obtain the same efficacy as
that obtained by the administration of recombinant peptides in
accordance with the present invention. These same results were
mirrored in the *in vivo* testing undertaken and illustrated in
Figs. 3 through 5.

13. A common way to measure hPTH bioactivity is
to determine its ability to activate cell membrane-bound
adenylate cyclase in target cells, e.g., bone derived cells.
When hPTH binds to its receptor, adenylate cyclase is
activated. This generates cAMP from ATP Mg. The activity
of adenylate cyclase can be directly measured in membrane
fractions of broken target cells when radioactive ATP Mg is
added and the radioactively generated cAMP is isolated and
quantitated by scintilation counting. The formation of cAMP

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over the course of a certain time period represents a measure of adenylate cyclase activity. The hPTH produced as described in my patent application exhibits full activity in an adenylate cyclase assay. Therefore, the recombinant hPTH possesses biological activity which is substantially equivalent to that of naturally occurring hPTH. Another method of determining biological activity is to measure the amount of cAMP generated inside intact target cells which have been treated with hPTH in the presence of an inhibitor of cAMP degradation. After a certain period of treatment, the reaction is stopped (the cells are killed) and cAMP is measured after extraction by radioimmunoassay. This is the test described in my paper in the journal *Peptides* (Fig. 2). Thus, the adenylate cyclase and the cAMP assays, both individually and collectively, establish the full biologicol activity of the hPTH I and my coinventors made.

14. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Dated: _02.29.96_

KAARE M. GAUTVIK, M.D.

F:\SS\DOCS\3.0\38429.DOC

6

CURRICULUM VITAE

Kaare M. Gautvik, professor dr.med.

Personal and marital status

Name: Kaare M. Gautvik
Home address: Bregnevn. 3, 0875 Oslo, Norway
Business address: Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112
              Blindern, 0317 Oslo, Norway
Telephones: 47-22851055 (work); 47-22235137 (home)

Date and place of birth: 11th of December 1939 in Oslo.
Social Security No.: 111239.39311

Married to: Vigdis Teig Gautvik, date of birth: 24th of March 1947

Children: Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964
             Silja Marie Sakrisvold Gautvik, date of birth: 31th of March, 1973
             Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education

1.   August 1958-June 64, Medical School at the University of Oslo.
2.   1967-69 Courses in mathematics involving
           geometry, statistics and mathematical analysis.
3.   May 1970, Disputation for the medical doctor degree at
           the University of Oslo.
4.   1985, Specialist in medicine, in clinical chemistry and physiology.

Employment

1.   June 1964 - June 1965, working at Tromsø University Hospital at medical and surgical
      departments.
2.   July 1965 until December 1965, working as a general practitioner in Sjøvegan, Troms.
3.   One year military service as a major in The Norwegian Air Force, working mainly at the
      Norwegian Institute for Aviation and Space Medicine.
      From 1967, position as post-doctoral researcher at The Institute of Physiology, Uni-
      versity of Oslo.

5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.

6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973 - 89.

7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.

8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
(At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).

Post-doctoral training abroad

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.

2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.

3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.

4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility

1. One year teching in aviation medicine for medical personnel and pilots.

2. I have given lectures and courses for medical students in following subjects: Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology. From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.

3. Organized interfaculty advanced courses within molecular endocrinology.

4. Lectures have been given in the following subjects at post-doctoral courses: Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).

5. Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.

6. Chief organizer of post graduate scientific courses for the Medical Fakulty at University of Oslo, 1986-1991.

7. Organizer of international scientific meetings within the frame of the following societies: Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.

8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.

9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below:

Examples of specially invited symposium lectures

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.

2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.

3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.

4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).

5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.crevisiae studied by in vitro mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.

6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).

7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Maccelesfield SK10 4TJ, UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal ß-oxydation, enzyme activities and mRNA levels".

8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning

and expression of human parathyroid hormone in microorganisms".

9.   Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology
       Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine
       Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could
       not attend).

10.   May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to
       held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biol-
       ogy".

11.   January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and
       Cellular Toxicology, Harvard School of Public Health and Department of Biological
       Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA.
       Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of
       G-coupled hormones".

12.   August 1993: Invited by Norwegian Society of Charted Engineers, The Blindern
       Conference. Lecture entitled: "Experience from industrializing basal research".

13.   November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at
       "Graduate course in molecular endocrinology - a problem oriented approach". The
       lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".

14.   February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH,
       Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in
       microorganisms and animal cells with special reference to signal sequence efficacy and
       intracellular modifications".

15.   September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology,
       Madurai Kamaraj University, India, to held a lecture in the symposium "Gene
       expression systems", XVIth IUBMB, New Delhi. Lecture entitled: "Expression of
       human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a
       milk protein in transgenic mice".

16.   November 1994: Invited by Professor A. Taschjian Jr., Harvard School og Public
       Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and
       functional characteristics of the human TRH receptor cDNA and mapping of the gene".

17.   February 11-13, 1995: Cairns, Australia, Workshop on "Animal Models in the
       Prevention and Treatment of Osteopenia"

18.   February, 1995: Int. Meeting of Calcified Tissue Research, Melbourne, Australia.

Honorary lectures and prizes

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".

2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.

3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.

4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.

5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.

6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.

Referee activity

I am or have been working as referee for the following international journals:

> Endocrinology
> J. Expl. Cell Res.
> Acta Physiol. Scand. (Kbh.)
> Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.)
> Eur. J. Clin. Invest.
> Hormone Research
> Acta Obstet. Gynecol. Scand.
> Journal of Endocrinological Investigation
> Eur. J. Biochem.
> Experimental Cell Research
> Scand. J. Gastroenterol.

Guidance for the academic doctor degree

1.   Veterinary, dr.lic. Richard Tollman: "Parturition hypocalcaemia in cows". 1976. Oslo.

2.   Dr.med. Trine Normann: "Medullary carcinoma of the thyroid. A morphological, clinical and experimental study". 1977. Oslo.

3.   Dr.med. Egil Haug: "Prolactin and growth hormone secretion by rat pituitary cells in culture. Hormonal control and mechanism of action". 1978. Oslo.

4.   Dr.med. Bjørn Klevmark: "Motility of the urinary bladder in cats during filling at physiological rates." Oslo. 1978.

5.   Dr.odont. Torill Berg Ørstavik: "Glandular kallikreins. Origin and secretion in some exocrine organs of the rat". 1978. Oslo.

6.   Dr.med. Arne Ekeland: "The role of calcitonin in fracture healing". 1981. Oslo.

7.   Dr.philos. Kjersti Sletholt: "Calmodulin from rat anterior pituitary tumour cells and its biological significance". 1988. Oslo.

8.   Dr.med. Trine Bjøro: "Regulation of prolactin secretion by hypothalamic hormone with special emphasize on vasoactive intestinal polypeptide (VIP)". 1988. Oslo.

9.   Dr.scient. Øyvind Andersen: "Purification and characterization of salmonid prolactin". 1989. Oslo.

10.   Dr.scient. Marianne Wright: "Biochemical studies of the pituitary receptor for thyrothropin-releasing hormone. Cell surface receptor protein characterization, receptor mRNA isolation and cDNA library generation and screening". 1991. Oslo.

      Dr.phil. Vendela Parrow: Signal transduction and gene regulation in cultured endocrine cells. 1991. Oslo.

11.   Dr.med. Eyvind J. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.

12.   Dr.philos. Ruth H. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.

13.   Dr.scient. Hilde Nebb Sørensen: "Actions of hormones and fatty acids on peroxisomal ß-oxidation enzyme activities and gene transcription." (Disputation 1993).

14.   Dr.philos. Najma Kareem: "The use of protein engineering to study hormone processing and secretion in different host cell systems". (Disputation 1994).

15.   Dr.philos. Ole Kristoffer Olstad: "Expression, purification and characterization of recombinant parathyroid hormone like peptides". (Disputation 1995).

16.   Dr.med. Berit Mortensen: "The influence of vit. D₃ on bone remodelling: In vitro and in vivo studies of bone turnover in the normal and uraemic conditions". (Disputation 1995).

17.   Dr.philos. Venke Skibeli: "Structural and functional aspects of Atlantic salmon growth hormone and prolactin". (Disputation 1996).

18.   Dr.med. Erik Rokkones: "Expression of heterologous peptide hormone genes in cultured cells and in animals". (Disputation 1996).

19.   Dr.philos. Sjur Reppe: "Secretion of heterologous proteins from the yeast Saccharomyces cerevisiae". (Disputation 1996).

Supervision of postgraduate candidates, thesis works:

I.   Cand.pharm. Per Wiik Johansen: "Regulation of prolactin and growth hormone secretion and synthesis by bromocriptine in rat anterior pituitary tumour cells". (Disputation 1996).

II.   Cand.scient. Vilborg Matre: "Cloning and expresjon of membrane receptors for hypothalamic hormones in exitable cells".

III.   Cand.scient. Hilde Hermansen Steineger: "Studies of regulatory gene-elements and transcription factores that mediate peroxisomal inducton and proliferation".

IV.   Cand.scient. Ole Petter Løseth: "Studies on hormonel bone remodulation in tissues and in animals".

V.   Cand.scient. Per Ivar Høvring: "Structure analysis and functional studies of cloned thyroliberin receptor and receptor isotypes".

VI.   Siv.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells".

Supervision of students' main degrees

1.   Cand.pharm. Åse Aulie: "The effect of somatostatin on cultures of growth hormone and prolactin producing cells." University of Oslo. 1979.

2.   Cand.pharm. Per Wiik Johansen: "The effects of bromocriptin on prolactin and growth hormone producing rat pituitary gland cells in culture." University of Oslo. 1981.

3.   Cand.pharm. Nina Lillegraven: "The significance of extracellular ion influence on the binding of thyroliberin to rat pituitary gland cells in culture." University of Oslo. 1982.

4.   Stud.med. Eyvind J. Paulssen: "The effect of TRH and oestradiol on prolactin-synthesis in rat pituitary cells in culture." University of Oslo. 1983.

5.   Cand.pharm. Kari Furu and cand.pharm. Kirsten Kilvik: "The uptake mechanism for oestradiol in rat pituitary cells in culture." University of Oslo. 1984.

6.   Cand.pharm. Berit Taranrød Johansen: "Cloning of mRNA for rat prolactin". University of Oslo. 1986.

7.   Cand.scient. Marianne Wright: "Characterization of surface proteins of GH-cells with special reference to the TRH receptor". University of Oslo. 1987.

8.   Cand.scient. Jenny Owe: "Binding and degradation of thyrotropin releasing hormone in hormone producing rat at pituitary cells in culture". University of Oslo. 1988.

9.   Cand.real. Grete Sørnes: "Effects of vitamin D on $^{45}Ca^{2+}$ efflux and prolactin production". University of Oslo, 1988.

10.   Cand.pharm. Siv Eriksen: "Development of an in solution mRNA hybridization test using antisense mRNA probes for prolactin". University of Oslo. 1988.

11.   Cand.real. Tom Skyrud: "Effects of human growth hormone and IGF-I on growth and clinical chemical plasma parameters". University of Oslo. 1988.

12.   Cand.pharm. Laila Northeim: "The inductive effect of tetradecyl-thio-acetic acid on peroxisomal ß-oxidation in 7800 C1 Morris hepatoma cells is stimulated by dexamethasone and inhibited by insulin". University of Oslo. 1988.

13.   Dipl.ing. Kristin Austlid Taskén: Transfeksjonsstudier i Karpe- og rotte hypofyseceller. University of Trondheim (NTH)/University of Oslo. 1988

14.   Cand.real. Hilde Nebb Sørensen: "The mechanism of ^{3}H-Dexamethasoneuptake into

7800C, hepatoma cells in culture". University of Oslo. 1989.

15. Stud.real. Najma Kareem: "Secretion and processing of recombinant hPTH in E.coli. Significance of preprosequences". University of Oslo. 1989.

16. Stud.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells.

17. Cand.scient. Even Sollie: "Stability of peroxisomal ß-oxidation enzyme activity and mRNA levels". University of Oslo. 1993.

18. Stud.scient Åse-Karine Fjelheim: Cloning and characterization of the human thyrotropin releasing hormone. (1996).

Guest research workers from abroad

In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.

Member of comittees for the academic doctor degree in Norway and abroad.

1. Opponent at dr.med. Bjørn Biber's disputation at Physiological Institute, The University of Gothenburg, Sweden. The work represented gastrointestinal physiology. 1974.

2. First opponent at dr.med.vet. Knut Hove's disputation. The work represented the effect of insulin on the intermediate metabolism in ruminants and mammary gland physiology. University of Tromsø, Norway, 1978.

3. First opponent at siv.ing. Kirsten Sandvig's disputation on the work: "Interaction of the toxic lectins abrin, ricin, and modeccin with mammalian cells". The work includes biochemical examinations on absorbtion and effect of toxic lectins in cell cultures. University of Oslo, Norway, 1979.

4. Opponent at cand.real. Anne Sundby's disputation on the work: "Plasma testosterone in young bulls in relation to age, gonadotropin stimulation and rate of weight gain and some studies on testicular gonadotropin receptors". University of Oslo, Norway, 1982.

5. Opponent at siv.ing. Anders Sundan's disputation on the work: "Studies on the entry of modeccin, diphtheria toxin, ricin, and pseudomonas toxin into mammalian cells". University of Oslo, Norway, 1985.

6. Opponent at dr.med. Svein Dueland's disputation on the work: "Absorption and transport of vitamin D_3 and 25-hydroxy-vitamin D_3 in the rat". University of Oslo, Norway, 1986.

7. Opponent at dr.philos. Dagny Sandnes's disputation on the work: "Beta-adrenoceptors on rat hepatocytes and human mononuclear leucocytes, with special reference to quantitation and regulation". University of Oslo, Norway, 1988.

8. Opponent at dr.med. Øyvind Sverre Bruland's disputation on the work: "Preparation and properties of two novel highly specific antisarcoma monoclonal antibodies and their application in the characterization and diagnosis of human sarcomas". University of Oslo, Norway, 1989

9. Opponent at dr.med. Pål Wiik's disputation on the work: "Vasoactive intestinal peptide as a modulator in the neuro-immune axis; the influence of stress". Norwegian Defence Research Establishment, Norway, 1989.

10. Opponent at dr.med. Eystein S. Husebye's disputation on the work: "Stimulus-secretion coupling in chromaffin cells of the bovine adrenal medulla. With special reference to the role of phospholipid metabolism". University of Bergen, Norway, 1990.

11. Opponent at dr.scient. Hooshang Lahooti's disputation of the work: "The estradiol receptor and the 90 kDa heat shock protein. Phosphorylation of the receptor and the heat shock protein, and studies on regulation of the estradiol receptor mRNA". University of Bergen, Norway, 1991.

12. Leading the disputation of Hilde Nebb Sørensen on the work: "Hormonal modulations of fatty acid stimulated peroxisomal ß-oxidation in cultured liver cells." University of Oslo, Norway, 1993.

13. Opponent at dr.odont. Janicke Liaaen Jensen's disputation on the work: "Human saliva: Biochemical and physiological aspects of some components", Faculty of Density, University of Oslo, Norway, 1994.

Member of advisory international/national committies for evaluation of professor positions.

1. Professor Ingrid U. Richardson, Harvard University, Boston, USA. 1974.
2. Professor Thomas F.J. Martin, University of Wisconsin, USA. 1984.
3. Professor Margaret A. Brostrøm, University of Medicine and Dentistry of New Jersey, USA. 1984.
4. Position as full Professor at the Institute of Physiolology, University of Gothenburg, Sweden. 1987.
5. Professor I at the Department of Clinical Chemistry, University of Tromsø, Norway, 1987.
6. Participation in an international board created by Sandoz, Basel, Switzerland, for nominating a candicate for the Sandoz International Endocrinological Prize in 1988.
7. Professor in Veterinary medicine, Norwegian Veterinary University, Oslo, Norway, 1989.
8. Position as Professor in Physiological Chemistry, University of Kuopio, Finland. 1989.
9. Position as Professor I in Endocrinology at the University of Gothenburg, Sweden, 1991.
10 Position as Associate Professor I in Endocrinology, University of Gothenburg, Sweden, 1993.
11. Position as Associate Professor I in Biochemistry, University of Bergen, Norway, 1993.
12. Appointed member of committy to evaluate chair Professorship at Karolinska Institution, Dept. of Endocrinology, Sweden (I had to decline because of sabattical year).

Honorary Societies

Member of the Norwegian National Academy of Science and Letters

Professional memberships

Norwegian Society of Biochemistry
Norwegian Society of Physiology
Norwegian Society of Endocrinology
Endocrine Society (USA)
Society for Calcified tissue (USA)
Society for Bone and Mineral Metabolism (USA)

Medical clinical specialities

1. Clinical Physiology and Chemistry including Nuclear Medicine
2. Work Medicine

Medical Faculty Responsibilities

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Reesearch Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's councel for evaluation of postgraduate applications from 1989-1993.

Research Council Responsibilities

1. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
2. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
3. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
4. Member of the International Scientific Board of Novo-Nordisk Research Committee.
5. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway.

Patents

General information

Two U.S. patents, U.S. Patent No. 5.010 010 and No. 5.420.242 are held with

international extensions in Europe, Japan, Canada, and Australia. In addition, three
Divitional Applications are submitted to the U.S. Patent Office and elsewhere.

All of Gautvik et al.'s patents and patent applications in the different countries are
covering specific methods related to the production, purification and characterization of
PTH in microorganisms.

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS GIVEN IN HISTORICAL ORDER:

I.   THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO PLASMA KININS AND KALLIKREINS

II. MOLECULAR ENDOCRINOLOGICAL RESEARCH

    A.   REGULATION OF HORMONE SECRETION AND SYNTHESIS

    B.   RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE HORMONE RECEPTORS

    C.   STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

III. ENDOCRINOLOGICAL RESEARCH RELATED TO HYPERFUNCTION OF THE PARATHYROID GLAND AND RESEARCH IN RELATION TO MEDULLARY CARCINOMA OF THE THYROID GLAND

    A.   CLONING AND EXPRESSION OF PARATHYROID HORMONE AND RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS AND TRANSGENIC ANIMALS

    B.   PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT HUMORAL HYPERCALCAEMIA

    C.   ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

    D.   ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

IV. MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

    A.   STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH

    B.   ISOLATION, PURIFICATION, AND CHARACTERIZATION OF S ALMON PROLACTIN AND GROWTH HORMONE

I.   THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO PLASMA KININS AND KALLIKREINS

Plasma kinins are biologically very active polypeptides that are distributed throughout the body and become accepted to be of importance for regulation of blood flow in certain organs. Kinin forming enzymes (kallikreins) and kinin inactivating enzymes (peptidases, "converting enzyme") have received new attention the last years due to their possible involvement in hypertension. My thesis from April 1970 dealt with certain physiological/biochemical aspects in relation to blood flow regulation including purification of substrates for kallikreins and their characterization in vitro. These components were used for studying their physiological interaction during perfusions of the salivary gland activated via nerve stimulation. By using purified substrates and their enzymes, a direct functional involvement of plasma kinins in functional vasodilatation could be demonstrated in vivo for the first time. Its title was "Studies on vasodilator mechanisms in the submandibular salivary gland in cats" (O.A.: 7).
Relevant references: R/C, 3,6,7,8,11,12,13; O.A, 1-6,8,9,11,16,19,23, 39,43,50, 55,56.

II.   MOLECULAR ENDOCRINOLOGICAL RESEARCH

A.   REGULATION OF HORMONE SECRETION AND SYNTHESIS

A major part of my research engagement has been carried out using functional cell cultures and transplantable tumors from highly differentiated cells that are able to perform organ specific functions. The following areas have been actively persued since 1971:

i.   The biological effects and mechanism of actions of the hypothalamic hormones thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide in prolactin and growth hormone producing rat pituitary cells.

These hypothalamic hormones are of central importance in regulation of release of prolactin and growth hormone from the anterior pituitary gland. The results which we have obtained with the cultured rat pituitary cells, have all been confirmed in more physiological endocrine model systems and thus appear to be valid for interpretation of how these regulatory hormones influence the functions of the anterior pituitary gland. I have carried out characterization of receptor binding of thyroliberin and been a senior researcher to originate research regarding receptor characterization for dopamine, somatostatin and vasointestinal polypeptide. In addition, I have steadily persued studies to elucidate and delineate the mechanisms of action for these peptide hormones. We have characterized the second messengers systems involving cyclic nucleotides and calcium, as well as described pathways of phospholipase C activation with formation of inositol triphosphates and

diacylglycerol. I have been one of the first in this research area to show the involvement of cyclic nucleotides and calcium in the action of thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide. How these second messengers were generated and their interaction, were first described in an invited review article for the Benzon Symposium, Copenhagen, 1988. (K.M. Gautvik et al., Regulation of prolactin secretion and synthesis by peptide hormones in cultured rat pituitary cells, Alfred Benzon Symposium 25, Copenhagen, 1988) and as an invited lecture at the 2nd European Congress in Ljubljana, 1990.

The ongoing research has concentrated on the involvement of GTP binding proteins in the receptor coupling of these hormonal signals as well as the characterization of the receptor itself. Thus we have identified and studied the functional coupling between these receptors and the G protein subunits in pituitary cells. Furthermore, these studies are now completed with a description of the adenylyl cyclase subclasses in the same cell-types and their engagement by the different hormone-receptor G protein subunits. These studies have involved measurements of specific mRNAs, the corresponding proteins and their regulation by the hypothalamic hormones as well as antisense RNA experiments testing the direct physiological involvement of G_{α} protein in the action mechanism of e.g. thyreoliberin.

ii)   The biological effects and mechanisms of action of steroid hormones (oestradiol, progesterone, testosterone, cortisone and vitamin D$_3$) examined in prolactin and growth hormone producing cells in culture.

Through the years 1973-1983, I was engaged in studying the effects of the above mentioned steroid hormones and characterization of their distinct receptors in prolactin and growth hormone producing cells in culture. We showed e.g. for the first time the existence of testosterone and vitamin D$_3$ receptors in adenopituitary cells. The biological effects of these hormones and how they regulate prolactin and growth hormone synthesis, were also examined in detail. How the steroid and polypeptide hormones regulated hormone receptor levels was studied during different conditions with the aim to understand their physiological interplay.

iii)   Regulation of rat prolactin and growth hormone gene expression in functional pituitary cells.

Many of the hormones which affects prolactin and growth hormone secretion are also able to change the rate of synthesis for these hormones. In the same decade, we developed immunopresipitation methods for the radioactively labelled hormones, and improved the sensitivity of hormone measurements to the level of single cells. This was shown using capillary tube gel electrophoresis of immunoprecipitated hormones and the results confirmed by concommitant immunocytochemistry. By using a combination of protein analysis and RNA blotting methods, we could show that prolactin synthesis was stimulated mostly by thyroliberin and oestrodiol while an inhibitory effect was found by dopamine and cortisone. The most efficient inducer of growth hormone synthesis was cortisone, and its

synthesis was also inhibited by dopamine.

Relevant references: O.A.: 12-14,22,24,25,27,31-34,36-38,42,44,48,49,51,57-59,62,66,
67,69,71-73,76,77,80,81,83-85,87,91,92,96-105,107,109,110,112,114,115,122,132,
133,134; R/C: 17,19,21,22,23,26,28-30,34.

B. ## RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE HORMONE RECEPTORS

In this respect, we have isolated and cloned cDNA for the receptor for thyroliberin in rats and in humans, the latter result as the first original description in the litterature. Prior to this work we characterized and visualized this receptor both by photo affinity labelling using the radioactive hormone as well as using a polyclonal antiserum made by us and raised against cell surface ideotypes. We cloned the rat and human receptor after making a mRNA based PCR product and a cDNA which was then used as probe for screening libriaries.

The human thyroliberin receptor, shows several interesting features when the aminoacid sequences are compared in rat and human. The differences have probably direct bearing on the functional activity of the receptors in relation to G protein coupling pattern as well as signal effector activation, which we in the rat have described in detail.

Relevant reference: O.A. 115,130,157.

C. STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The hypothalamus consists of discrete nuclei which paly a vital role in several biological functions that are essential to mammals and related to different homeostatic mechanisms, reproduction, behavior, emotion and responses to various stress. The hypothalamic nuclei in part, integrate many autonomic regulatory systems whose final path is expressed by neuroendocrine cells. Thus, they represent "high command centers" within the endocrine system and enable the central nervous system to initiate, adjust and balance intricate and complex endocrinological reflexes. This part of hypothalamic function is exerted by synthesis and release into the pituitary portal system of a number of substances, mainly of peptide and amine nature. These substances control the anterior pituitary gland function. In addition, hypothalamus is the site of production of two hormones, oxytocin and vasopressin, which are transported by axon flow into the posterior pituitary from which they are released into the general circulation affecting salt/water balance and being of

importance for parturition and breast feeding. Furthermore, the hypothalamus is a center for social and sexual biological behaviors and mediates feeding and drinking habits. The importance of hypothalamic functions in the field of calorie balance may be exemplified by the recent discovery by Zhang et al. (Nature, 372, 1994) who describe the first cloning of the so called mouse "obese" gene and its human analog, where the receptor for this hormone by all criteria has to be localized in a hypothalamic nucleus. Since the first objective of this research has been obtained, namely to generate a subtracted hypothalamic specific cDNA library of high quality, we will expect to find this receptor among the 10^5 independent clones present in the library, as well as clones of mRNA encoding a large number of other novel proteins.

The subtraction library shows inserts with sizes estimated on agarose gel electrophoresis between 0.4 and 1.2 kb (mean > 0.7 kb), a result which was very satisfactory.

The quality of the library is further assessed by the extent to which clones of certain mRNAs known to be present in hypothalamus had been amplified during subtraction, and to what extent clones of mRNAs which are ubiquitously present in the central nervous system had been removed. Vasopressin which is exclusively present in hypothalamus is enriched 20-30 times in the subtracted library compared to the hypothalamus cDNA library, and the commonly present NSE and cyclophilin is completely removed after subtraction. 215 clones from the subtracted library have been picked into grids and hybridized with probes prepared by PCR amplification of the inserts from the driver, target and subtracted libraries. Approximately 1/4 of the clones give substantially greater signals with the subtracted target probe than the unsubtracted target probes and faint or undetectable signals with the driver probe. If validated, these figures suggest that roughly 1% of the hypothalamus mRNA mass is enriched in that structure (corresponding to an estimated 300 different gene species, given that 30,000 species are expressed in the brain).

Conclusion: The results so far from our learning and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method are summarized. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. One article on the general aspects of hypothalamic enriched/specific mRNAs is in preparation. Another article is describing a novel somatostatin-like peptide, called cortistatin. This article is in press 1996 in Nature.

III. **ENDOCRINOLOGICAL RESEARCH RELATED TO HYPERFUNCTION OF THE PARATHYROID GLAND AND RESEARCH IN RELATION TO MEDULLARY CARCINOMA OF THE THYROID GLAND**

This research started out in the early 70'ies as a result of my development of two radio-immunoassays for parathyroid hormone and calcitonin, methods which at that time did not exist in Norway. Since 1973, I have thus carried out clinical laboratory diagnostic activity

for the whole country, and also received samples from other Scandinavian countries as well as England. In different collaborative studies, we used these assays in basal and clinical endocrinological research related to how these hormones were regulated by calcium both in vitro and in vivo. In addition, biochemical work was carried out in order to characterize different intracellular hormone-forms retrieved from tumor cells producing these peptides.

The hyperfunction of the parathyroid glands occurs in relation to development of adenomas and/or hyperplasia. The cause(s) of primary hyperparathyroidism is (are) unknown, while secondary hyperparathyroidism occurs as a result of chronically lowered serum Ca^{2+} (e.g. in chronic renal failure). The question about how low concentration of serum Ca^{2+} may induce not only increased hormone secretion and synthesis, but also trigger DNA replication and cell proliferation, is intriguing, but still unknown. We have studied patients with secondary hyperparathyroidism during various experimental conditions to address these questions. From human adenomas we isolated poly(A)$^+$ RNA for cloning of parathyroid hormone (PTH) in 1983.

Medullary carcinoma of the thyroid gland (MCT) occurs as an inherited and spontaneous malignant disease. As the first to introduce diagnostic tool to discover this calcitonin producing tumor in Norway, we have mapped the extent of the disease in our country.

Relevant references: O.A.: 28-30,40,41,52-54,61,65,68,70,74,78,79,82,86, 90,94,108, 117; R/C: 5,15,16.

A. ## CLONING AND EXPRESSION OF PARATHYROID HORMONE AND RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS AND TRANSGENIC ANIMALS

This project started in 1983 and developed into a major research engagement where we have succeeded as the first in the world, to clone and express this hormone in 100 mg quantities both as a product in E.coli as well as in Saccharomyces cerevisia. We have also successfully transfected mammalian cell cultures, insect cells and transgenic animals, silkworm larvae and mice, and obtained expression of this hormone. We have also expressed mutated forms in yeast and studied the intact hormone and fragments in insect cells and Bombyx mori larvae with the aim to understand cellular processing, traficking and secretion.

In the first part of the work we cloned the cDNA for human parathyroid hormone using conventional cloning techniques and expressed the peptide as met-gly product in E.coli. This peptide analogue was not biological active. In a search for peptides with antagonistic action, we found that gly PTH (1-84) was an interesting form having binding properties and inhibited the agonist by 40 per cent at $10^{-9}M$. In clinical medicine an effective PTH antagonist will be of potential use both as a diagnosticum and in treatment of hypercalcaemia.

For expression in S.cerevisiae recloning of the cDNA was carried out using a fusion construction with the prepro region of the yeast mating factor α gene. We were able to obtain an effective and correct N-terminal processing and isolated human PTH as a secretory product in a yield (up to 10 mg/l). We also developed a down-stream technology for purifying the hormone which was shown to be identical to the authenthic peptide hormone by a variety of chemical, biochemical and biological test systems.

In order to obtain a higher yield of human parathyroid hormone, we in vitro mutagenized a proteolytic cleavage site internal to the peptide, and obtained a full-length agonist (84 amino acids) which after purification was shown to have the same biological activity as the authentic hormone.

A part of this work has been concentrated on to find optimal signal sequences both for expression in E.coli as well as in yeast, where we by using different amino acid substitution in new gene constructs, have developed an effective test system for looking at N-terminal processing. This is obtained by making a fusion gene between the N-terminal region of the PTH gene and the protein A gene which in E.coli is transcribed using the protein A promoter and transcription stop signals.

We have also made constructs for use in mammalian cells where we employ the Whey Acidic Protein (WAP) promoter region in order to express PTH in mammalian cells of mouse origin. We have recently in addition generated transgenic mice who express PTH as a secretory product in milk.
Relevant references: O.A.: 124,128,129,133,139,140,144,145,161,162,163,166, 167,168; R/C: 31,32,35.

B.   PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT HUMORAL HYPERCALCAEMIA

Parathyroid hormone related peptide has been isolated as the causative agent during conditions of malignant humoral hypercalcaemia. This condition occurs in the presence of several malignent diseases such as cancers, carcinomas as well as myelomatosis where the tumour cells are able to produce a parathyroid hormone related peptide. We have received cDNA clones and recloned it in yeast for production of the protein to be used in receptor binding- and activation-studies. We are also studying the expression of this gene in animal and human tumour cells, with the purpose to learn gene specific splicing.
Relevant references: O.A.: 151; 160, Jemtland et al., Rian et al. submitted; R/C: 36.

C.   ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described in

Chapter II,C, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual cones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumortype.

D. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of sentral importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular substraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is in its initial phases.

IV. MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

A. STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH

The endocrinological aspects as it relates to regulation of growth and development of fertility, are as important in fish as in mammalian species. In addition, fish is an i nteresting model system also for studies within embryology, differentiation, and gene regulation. As in mammalian species, growth hormone will regulate growth and prolactin will be of importance for normal fertility and adaptation to salt/fresh water conditions. Also, in this research area, we have worked partly from the protein side and partly from the DNA side. We have as first reports described isolation and purification of prolactin and growth hormone from Atlantic salmon, and developed sensitive radioimmunoassays in order to follow the hormones in fish as a function of age and also during different experimental conditions.

The initial DNA work for production of transgenic fish is published and may be summarized briefly:

We first developed a new microinjection technique where small amount of foreign DNA was injected into fertilized fish eggs and the survival rate was more than 90%.

As the first model gene we used the human growth hormone gene (kindly given from Professor R. Palmiter, USA) where the promoter for the metallothionin gene ensured expression in eukaryotic organisms. From this gene construction, we made cDNA probes which specifically hybridized to growth hormone DNA and mRNA.

The microinjected DNA for human growth hormone gene incorporated in the embryo's chromosomal DNA was isolated and demonstrated by Southern blot analysis. It was incorporated in the fish genome already after 7 days.

We also showed that the human growth hormone gene was active based on the occurrence of specific mRNA for human growth hormone and production of growth hormone by the fish embryo and secretion to the medium. This achievement shows that it is possible to develop a rather unique model to study gene expression both under embryonic development and in the adult fish (R/C 44; O.A. 119, Skibeli submitted 1996).

In order to measure expression of Atlantic salmon growth hormone, we have isolated and purified this hormone as well as prolactin from the same species. (O.A. 118, 121, 135, 138).

B. ISOLATION, PURIFICATION AND CHARACTERIZATION OF SALMON PROLACTIN AND GROWTH HORMONE

At the time we started out this project, a preliminary sequence of the corresponding hormones in the Pacific salmon was known. During this project we were however, able to purify and characterize both these hormones from the Atlantic salmon and were the first to give the amino acid sequence data on both these hormones. In addition, we used our own made prolactin antisera for studying the possible function and involvement of prolactin in sex maturation of Atlantic salmon. Growth hormone was further characterized by detailed chemical and biochemical analysis including phosphorylation and glycylation patterns, development of antisera against fragments of the hormone, and analysing the immunoreactivity of growth hormone from different salmon species. These reports describes for the first time GH species as two gene products in Salmon fish, and they are both glycoproteins, and one also phosphoglycoprotein.

Relevant references: R/C 44; O.A. 118, 119, 135, Skibeli submitted 1996).

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 4 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I.   PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

i)   Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.

ii)   Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

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2.   Gabrielsen OS, Reppe S, Sletten K, Øyen TB, Sæther O, Høgset A, Blingsmo OR, Gautvik VT, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and secretion of human parathyroid hormone in Saccharomyces cerevisiae. Gene, 90(2): 255-262, 1990.

3.   Høgset A, Blingsmo OR, Sæther O, Gautvik VT, Holmgren E, Josephson S, Gabrielsen OS, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and characterization of a recombinant human parathyroid hormone secreted by E.coli employing the staphylococcal protein A promoter and signal sequence. J. Biol. Chem., 265: 7338-7344, 1990.

In this regard we have received acceptance for an international patent on gene constructions, plasmids, the process and the down-stream technology.

In the further work we have by using in vitro mutagenesis, created full length parathyroid hormone agonist which has shown to be protease resistant and have interesting biological actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

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In our ongoing studies regarding the mapping of functional domains in human parathyroid hormone in comparison with the parathyroid hormone-like protein (PTHrP) we have expressed the human forms successfully in Saccharomyces cerevisiae and have also cDNA clones for their receptor as well as permanently transfected mammalian cells which express the receptor on the surface. By having access to PTH and PTH analogues as well as PTHrP, we are in a good position to map out the binding affinities of different hormonal forms as well as their coupling to different cellular signal systems.

Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding K_D which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.

9. Rian, E., Jemtland, R., Olstad, O.K., Gordeladze, J.O., Gautvik, K.M. Expression of biologically active human parathyroid hormone-related protein (1-141) in Saccharomyces cerevisiae. Eur. J. Biochem. 213: 641-648, 1993)

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The various recombinant PTH and PTHrP forms will be studied in their interaction with natural receptors of bone cells in culture and also in relation to recombinant receptor permanently transfected in mammalian cells. The aim of this is to understand in more detail the structure activity relationship between the different hormonal forms and their ability to activate different cellular signalling systems. The ultimate goal will be to try to understand how the osteoblast is activated by parathyroid hormone in the bone remocelling process of importance for the elucidation of the causes and patogenesis of osteoporosis.

II. NEUROENDOCRINE RECEPTORS AND THEIR FUNCTION

During our cDNA cloning of G-coupled receptors in rat pituitary cells and in human CNS, we have identified four potential candidates for G protein coupled receptors distinct from the TRH clone in a human phage library. We were able to isolate and characterize a functional human TRH receptor and to present these results as the first original international report. In addition to engaging in characterization of the other receptors, we are at present working on the organization and functional aspects of the gene for the human TRH receptor.

The ongoing and future research will concentrate on:

1. To map deleted receptor cDNA clones for functional activity using the Xenopus laevis oocyte system as a hormone (TRH) specific bioassay.
2. Generation of transfected cells to map out the hormone-binding receptor region as well as dissect which part of the hormone receptor couples to the two previously characterized G proteins which mediate signal system activation conveying its physiological actions, namely the $G\alpha s$ coupling to the adenylyl cyclase system and the Gq/11 coupling to the phospholipase C.
3. Making hybrid receptors between the thyroliberin and the PTHrP/PTH receptor in order to analyze further the importance of the different receptor domain for conveying signal transduction.
4. Analyse the 5'-end of a newly isolated genomic clone for the human receptor.

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III. STUDIES OF THE INTERACTION BETWEEN FATTY ACIDS AND
DEXAMETHASOME AND INSULIN REGARDING REGULATION OF
PEROXISOMAL ß-OXIDATION ENZYMES. POSSIBLE INVOLVEMENT OF
THE PEROXISOMAL PROLIFERATOR ACTIVATED RECEPTOR (PPAR)
GENE AND ITS REGULATION

On a collaborative basis within my institute, I have since 1991 engaged in research
regarding hormonal control of peroxisomal ß-oxidation enzymes triggered by a surprising
finding that fatty acids and dexamethasome have strong synergistic actions in regulation of
the transcription of these three enzyme genes. This positive cooperativity was completely
blocked by insulin in cultured liver cells and also in intact rats. The studies were carried
out on the RNA, protein and enzyme activity levels and opened a new side of this already
very much studied area of fatty acid ß oxidation. The ongoing research in this area will
continue as a collaborative work between professor Jan-Åke Gustafsson's group at
Huddinge Hospital and Institute for Medical Biochemistry, where we will concentrate on
delineating the possible regulatory elements located in a genomic clone of the PPAR from
rat.
Relevant O.A.: 120,125,127,131,147,150.

IV THE USE OF A NOVEL SENSITIVE MOLECULAR SUBTRACTION
HYBRIDIZATION METHOD FOR STUDYING DIFFERENTIALLY
EXPRESSED mRNAs

Studies of hypothalamic specific mRNAs obtained by subtraction hybridization procedure

Isolation and characterization of mRNAs specific for the osteosarcoma phenotype obtained
by subtraction hybridization

Isolation and characterization of mRNAs psecific for parathyroid hormone gene activation
in bone cells

Conclusion

This ongoing work has very successfully been able to isolate unique hypothalamic specific
mRNAs among those also a novel somatostatin-like peptide. In addition, this subtracted
library will probably contain the long sought for receptors which fatty acid or their
metabolites are acting on, in order to regulate calorie intake and consumption. This
bridges then the research going on in Chapter III and its work. The unique subtracted
hypothalamic library can be exemplified with the finding that we also have isolated a novel
calcium calmodulin kinase whose distribution is unique in CNS and also a transmembrane
protein of secretory vesicles which has never previously been cloned in mammalian
species, but has its homology in the electric organ of the electric eel. The characterization
and studies of full-length cDNA clones from these mRNAs are given highest priority.

Already the differential display of mRNAs present in human osteosarcoma cells and absent in normal bone cells is very promissing and certainly leads to encouraging considerations regarding the possibility to obtain osteoporosis specific genes as defined by PTH specific mRNAs in normal osteoblasts.

P U B L I C A T I O N   L I S T

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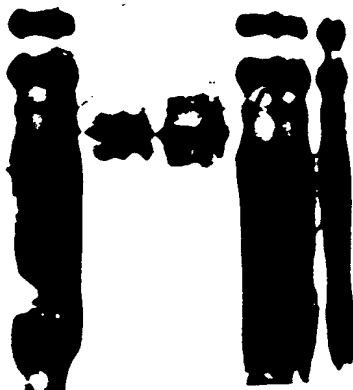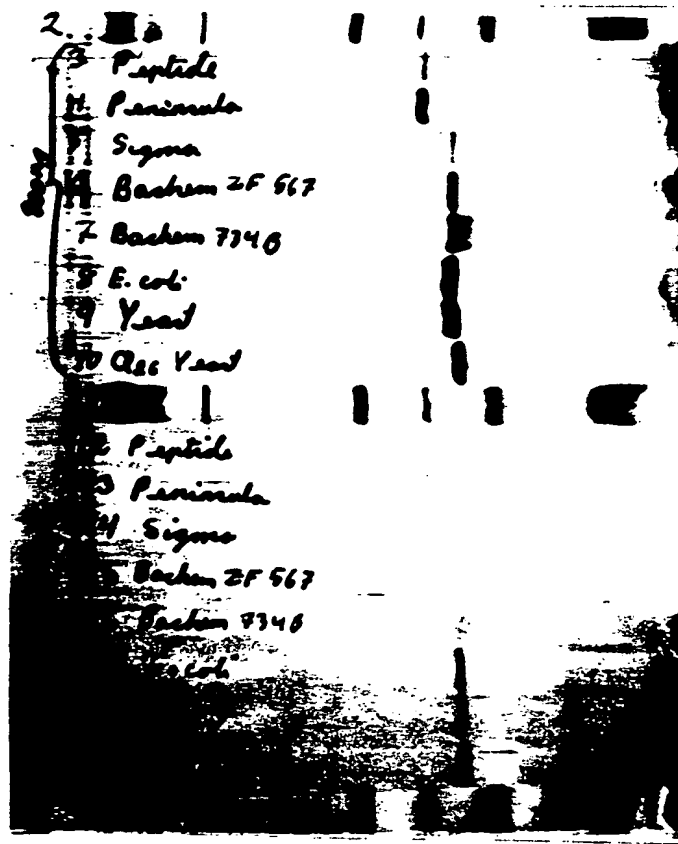
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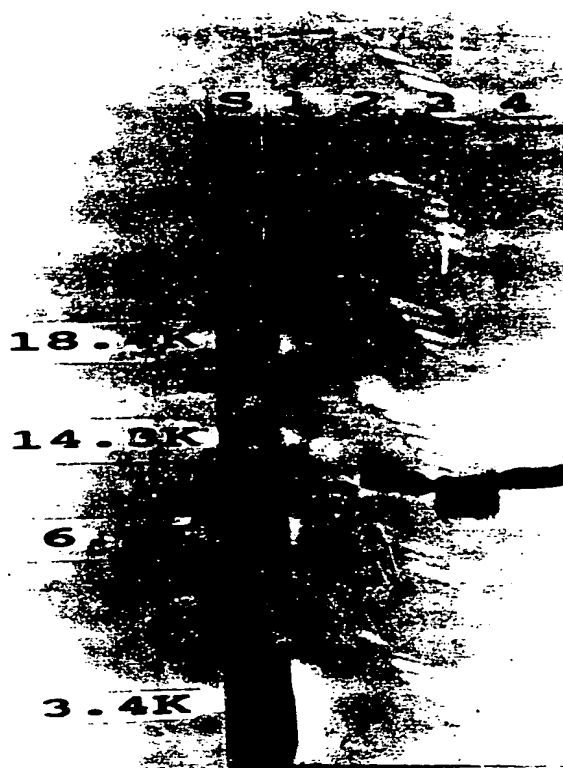
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Differences in Binding Affinities of Human PTH(1-84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms

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OLSTAD. O. K.. N. E. MORRISON. R. JEMTLAND. H. JÜPPNER. G. V. SEGRE and K. M. GAUTVIK. *Differences in binding affinities of human PTH(1–84) do not alter biological potency: A comparison between chemically synthesized hormone. natural and mutant forms.* PEPTIDES 15(7) 1261–1265. 1994.—The purpose of this study was to evaluate receptor binding affinities and biological properties in vitro and in vivo of various recombinant hPTH(1–84) forms representing the natural hormone and a mutagenized hPTH form, [Gln^{26}]hPTH(1–84) (QPTH). after expression in *E. coli* and *Saccharomyces cerevisiae*. In LLC-PK$_1$ cells stably transformed with the rat PTH/PTHrP receptor. chemically synthesized hPTH(1–84) and QPTH showed a reduced binding affinity (apparent K_d 18 and 23 nM. respectively) than the recombinant. hPTH(1–84) (apparent K_d 9.5 nM). All recombinant hPTH forms showed a similar potency to stimulate cellular cAMP production (EC$_{50}$ 1.5 nM) and significantly better than chemically synthesized hPTH (EC$_{50}$ 5.7 nM). All hormone forms showed an about equipotent activity in causing elevation in serum calcium. increased excretion of urine phosphate. and cAMP. Thus. the natural recombinant PTH forms showed higher binding affinities and adenylate cyclase activation potencies in LLC-PK$_1$ cells. but the reduced receptor binding affinity exerted by QPTH did not transcend differences in cAMP generation and in vivo biological activities.

Recombinant parathyroid hormones   Recombinant PTH/PTHrP receptor   cAMP response   Rats

PARATHYROID hormone is the principle regulator of calcium homeostasis in humans and has been advanced as an anabolic drug against postmenopausal osteoporosis (22,25). The hormone. which is produced in the mammalian parathyroid glands. is synthesized as an 115 amino acid precursor that is processed to the mature hormone of 84 amino acids (21). The information required for high-affinity binding of PTH to its receptor in bone and kidney cells is contained within the biologically active 1–34 region (20). The amino-terminus of PTH is essential for triggering the adenylate cyclase response pathway (8.26). but it also contributes modestly to receptor binding affinity. In addition to a nearly complete loss of cAMP agonism. the deletion of residues 1–6 is accompanied by an approximately 100-fold decrease in receptor binding affinity (7,10,18,24). The major component of PTH receptor binding affinity. however. appears to be determined by residues 28–34. Deletion of these residues causes at least a 1000-fold reduction in binding affinity (18). Furthermore.

PTH(25–34) displays weak. but detectable. receptor binding affinity ($K_d \approx 100 \mu M$) (18). In comparison. no evidence for receptor interaction has been obtained for amino-terminal fragments shorter than PTH(1–27) (24.26). Based on these observations. the 25–34 region has been called the hormone's principal receptor binding domain (18).

We have previously reported production of hPTH(1–84) in yeast (5). and the α-factor expression system is a well-characterized. commonly used strategy for expression of foreign proteins by the yeast *Saccharomyces cerevisiae* (3.27.29). The mating factor alpha (MFα) leader sequence is cleaved off sequentially by the KEX-2 endopeptidase and then by an amino peptidase STE13. leaving a correct *N*-terminal after guiding the recombinant protein through the secretory pathway (11). In the expression plasmid pαUXPTH-2. the MFα promoter. signal sequence. and termination signal were employed. The secreted hormone was purified from medium to more than 95% homo-
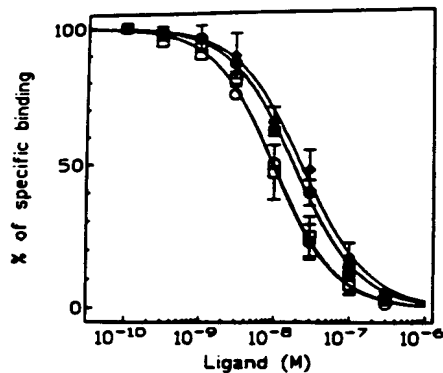
FIG. 1. Inhibition of radiolabeled [Tyr[36]]chickenPTHrP(1–36)amide by different hPTHs. Recombinant hPTH(1–84) produced in *E. coli* (O), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●) were tested in radioreceptor assay using LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean ± SD of at last two independent experiments, each performed in triplicate.

geneity, characterized chemically, and shown to represent the natural hormone (5,19). In addition to the intact hormone, an aberrant KEX-2 cleavage occurring at an internal site (5) after two consecutive basic amino acids in the hPTH sequence -Arg[25]-Lys[26]↓Lys[27]-resulted in part fragmentation of the hormone. To improve the yield of hPTH, and to avoid internal degradation, a point mutation was introduced into the gene, changing Lys in position 26 to Gln (Q) (23). The resulting agonist, [Gln[26]]hPTH(1–84), called QPTH, was tested together with recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* and compared with chemically synthesized hPTH(1–84) in certain biochemical and biological tests.

We have also produced full-length hPTH in *E. coli* as a secretory product employing the *Staphylococcus aureus* protein A signal and regulatory sequences (9). After purification from
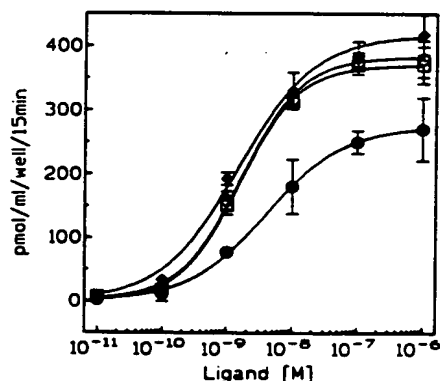
FIG. 2. Stimulation of cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor stimulated (15 min, 37°C) with recombinant hPTH(1–84) produced in *E. coli* (O), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●). The data represent the mean ± SD of two independent experiments, each performed in duplicate.
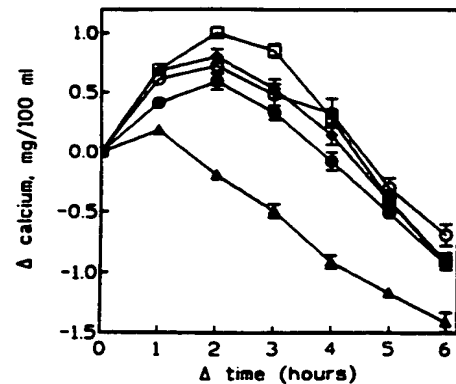

FIG. 3. Induction of hypercalcemia by different hPTHs. Parathyroidectomized male Wistar rats were administrated different forms of hPTH (2.0 μg of the recombinant hPTHs, 2.7 μg of the chemically synthesized hPTH) as described in the Method section. The stimulating agents were recombinant hPTH(1–84) produced in *E. coli* (O), recombinant hPTH(1–84) produced in *Saccharomyces cerevisiae* (□), [Gln[26]]hPTH(1–84) (QPTH) produced in *Saccharomyces cerevisiae* (♦), and chemically synthesized hPTH(1–84) (●). Control (▲). Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 h after injection of PTH. The results are reported as the difference between the amount of calcium in the blood at the various time points, subtracting out the amount of calcium in the baseline sample (delta values). The data represents the mean ± SEM (n = 6).

medium and chemical characterization, this recombinant form was also included in the biochemical and biological characterizations.

LLC-PK₁ cells (porcine renal epithelial cells) stably transfected with the cDNA for the rat PTH/PTHrP receptor (4) were used for the receptor binding studies and cAMP responsiveness; rats
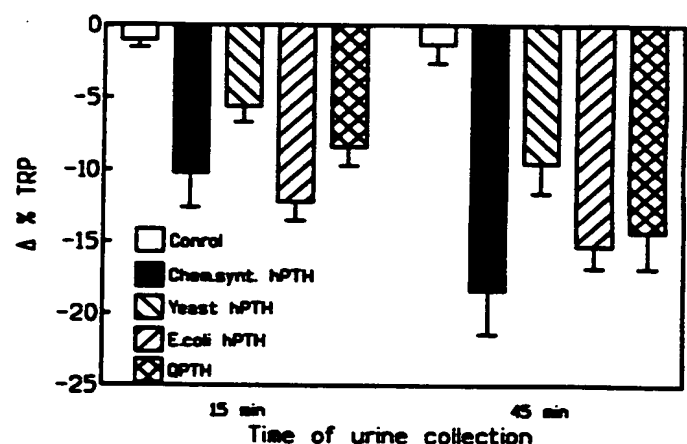

FIG. 4. Urinary excretion of phosphate. Parathyroidectomized male Wistar rats were administrated different forms of PTH (2.0 μg of the recombinant hPTHs, 2.7 μg of the chemically synthesized hPTH) as described in the Method section. Urine was collected for two periods: 0–30 and 30–60 min after administration of PTH. The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP) and is calculated by the formula: (1 − phosphate clearance/creatinine clearance) × 100. The result is reported as a change in % TRP related to the zero control level, and a decrease represents a greater amount of phosphate excreted in the urine. The data represents the mean ± SEM (n = 6)
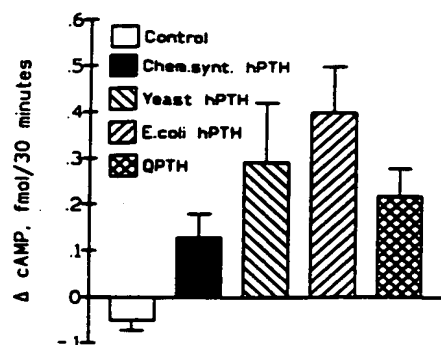
FIG. 5. Changes in urinary cAMP after administration of PTH. Parathyroidectomized male Wistar rats were administrated different forms of PTH (2.0 μg of the recombinant hPTHs, 2.7 μg of chemically synthesized hPTH) as described in the Method section. Urine was collected for 30 min after administration of PTH. The excretion of cAMP is reported as a change in cAMP concentration related to the zero control level. The data represents the mean ± SEM (n = 6).

were used for measurements of the hypercalcemic response, urine phosphate, and cAMP.

METHOD

Chemically synthesized hPTH(1-84) was purchased from Bachem Fine Chemicals (Torrance, CA) and [Tyr^{36}]chicken-PTHrP(1-36)-NH$_2$ for radioiodination was from Peninsula Laboratories. The production, purification, and chemical characterization of recombinant PTHs have been described previously (5,9,19,23). Peptide concentrations were determined by amino acid analysis. The blood and urine samples were analyzed for calcium, phosphate, protein, and creatinine on the Cobas Bio Autoanalyzer. cAMP was analyzed using a commercial radioimmunoassay kit from Amersham. All reagents were of highest purity available.

Radioreceptor Assay

LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (4) were plated in 24-well plates. The cells were incubated with ^{125}I-labeled [Tyr^{36}]chickenPTHrP(1-36)-NH$_2$ (100.000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris-HCl), pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal calf serum as described (28). The competing ligands included chemically synthesized hPTH(1-84) from Bachem, recombinant hPTH(1-84) expressed in E. coli (9), recombinant hPTH(1-84) expressed in yeast (5,19), and recombinant QPTH expressed in yeast (23). Techniques used for radioiodination of PTHrP analogue have been reported (12.13). PTH and PTHrP bind to and activate PTH receptors in bone and kidney in an indistinguishable manner (12,14). [^{125}I][Tyr^{36}]chickenPTHrP(1-36)-NH$_2$ was used as ● ligand because of lower nonspecific binding (less than 5% ●al binding) (14) compared to [^{125}I][Nle^{8,18}Tyr^{34}]bovine-●(1-34)-NH$_2$, which gave 10-15% nonspecific binding (28).

Intracellular cAMP Measurements

For measurements of intracellular cAMP, LLC-PK$_1$ cells (4) expressing the rat PTH/PTHrP receptor were pleated in 24-well ●lates (50.000 cells/well) and grown to confluence for 3 days

(about 250.000 cells/well). The cells were placed on ice, rinsed once with 1 ml of cold Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min. Then the cells were rinsed once with 0.5 ml phosphate-buffered saline and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 N HCl.

Hypercalcemic Assay

Male Wistar rats (150-200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. Preliminary experiments showed that this was a reliable way to obtain complete removal of parathyroid gland activity because plasma calcium fell linearly as a function of time, as also indicated by the control group in Fig. 3. Moreover, individual rats showed small variations in the results. The parathyroid glands were removed for two reasons. One, to eliminate the endogenous production of the hormone, and two, to make the animals more sensitive to exogenous hormone. The increase in sensitivity is assumed to be due to the upregulation of PTH receptors in target organs (16). Thus, it has previously been shown that tubular membranes prepared from parathyroidectomized rats reveal a higher binding of [^{3}H]hPTH(1-34) and higher maximum stimulation of PTH-stimulated adenylate cyclase compared to control animals (16). It has also been shown (28) that downregulation of PTH receptors in ROS 17/2 cells occurs when the cells are exposed to PTH concentrations near hormonal physiological doses. More than 50% of the of the PTH-stimulated adenylate cyclase activity was recovered within 24 h after desensitization.

The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4 % bovine serum albumin, 25 units heparin/ml. Five minutes after injection of 200 μl of the heparinized Ringers acetate, a baseline blood sample was drawn (300 μl). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH agonist was then injected SC in a volume of 200 μl. All agonists were dissolved into 100 μl of 0.01 N acetic acid. The test agents included:

1. vehicle, 0.001 N acetic acid, 1% bovine serum albumin (control),
2. chemically synthesized human PTH(1-84), 2.7 μg/rat (chem. synt. hPTH),
3. recombinant human PTH(1-84) from yeast, 2.0 μg/rat (yeast hPTH),
4. recombinant human PTH(1-84) from E. coli, 2.0 μg/rat (E. coli hPTH),
5. recombinant [Gln^{26}]hPTH(1-84) from yeast, 2.0 μg/rat (QPTH).

Due to the reduced receptor binding affinity and cAMP stimulation in the in vitro assays below, the chemically synthesized hPTH concentration was used at 2.7μg/rat.

After dissolving in acetic acid, the agents were brought up in 900 μl of Ringers acetate containing 1% bovine serum albumin. Blood samples were drawn at 1, 2, 3, 4, 5, and 6 h after injection ●f hPTH ●r ●g●n●st. The r●ts ●●ere ●●●h●n●●ni●ed 5 min ●ef●re

drawing each blood sample using 200 μl of the heparinized Ringers solution.

All forms of hPTH were analyzed and quantified by amino acid analysis before administration to the rats.

The blood samples were centrifuged in a clinical centrifuge for 10 min. then the plasma was analyzed for calcium using a Cobas Autoanalyzer.

Urine Analysis

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. The animals were fasted overnight. and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery and the jugular vein were cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate. 4% bovine serum albumin. 25 units heparin/ml. The bladder was catheterized using PE-200 tubing.

The carotid artery was cannulated for the collection of blood samples. and the jugular vein was cannulated for the purpose of injecting the hormones. and for a slow infusion for the purpose of volume loading the rats to increase the urine output. The rats were infused with Ringers acetate. 4% bovine serum albumin at the rate of 3 ml/h. The infusion was run for 2 h before the start of the experiment to equilibrate the animals.

After the 2-h equilibration period. a baseline urine collection was made for 30 min. with a midpoint arterial blood sample drawn at 15 min. At the end of the baseline urine collection. the PTH was injected IV. and a new 30-min urine collection was started. Again. a midpoint blood sample was taken 15 min into the urine collection. A final 30-min urine collection was made from 30–60 min after the injection of PTH. with the midpoint blood collection made at 45 min after PTH injection.

The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP). The % TRP is calculated by the formula: $(1 - \text{phosphate clearance/creatinine clearance}) \times 100$. A decrease of % TRP represents a greater amount of phosphate excreted in the urine. The creatinine clearance did not change in any of the treatment groups. It was not expected to change. and was only measured to calculate the % TRP.

Statistical Analyses

A two-sample t-test was used comparing mean values of control and treated groups of animals (2).

RESULTS

Radioreceptor Binding Studies and Intracellular cAMP Measurements

Binding of the different hPTH forms is shown in terms of displacement curves using the $[^{125}I][Tyr^{36}]$chicken PTHrP(1–36)-NH₂ as radioligand and LLC-PK₁ cells permanently transfected with the rat PTH/PTHrP receptor.

The chemically synthesized hPTH and QPTH had calculated binding affinities with K_d of 18 nM (95% confidence interval: 16.1–20.0 nM) and 23 nM (95% confidence interval: 19.0–27.2 nM). respectively (Fig. 1). The natural recombinant hPTH(1–84) forms from *Saccharomyces cerevisiae* and *E. coli* had a similar but significantly lower apparent K_d of 9.5 nM (95% confidence interval: 8.7–10.4 nM) (Fig. 1). In spite of these differences in receptor binding affinities. all the recombinant hormones had equal ability to stimulate intracellular cAMP accumulation (EC_{50} about 1.5 nM. 95% confidence interval: 1.0–2.2 nM) (Fig. 2). In contrast. the synthetic hPTH showed a significant reduced potency to stimulate cAMP production with EC_{50} of 5.7 nM

(95% confidence interval: 3.4–9.6 nM) on a molar basis. and a reduced maximal response.

Hypercalcemic Assay

After parathyroidectomy. the control calcium concentration fell linearly 1 h after the operation (about 0.75 mg %/h) (Fig. 3). The chemically synthesized hPTH was injected in a dose of 2.7 μg/rat compared to 2.0 μg/rat employed for the other hPTH species due to the reduced receptor binding affinity and cAMP stimulation of the chemically synthesized hPTH. These concentrations were chosen on the basis of preliminary experiments using a range of different doses and were selected because they gave a healthy hypercalcemic response and no observable side effects (e.g.. unaffected rectal temperature). The hypercalcemic response of the chemically synthesized preparation was somewhat lower than for yeast hPTH. but almost similar to the other recombinant hPTHs. From these experiments it appeared that the declining parts of the curves were similar and like the slope of the control curve (Fig. 3).

Tubular Reabsorption of Phosphate

The percent tubular reabsorption of phosphate (% TRP) was calculated on basis of urine creatinine values and showed a strong and significant ($p < 0.01$) reduction after injection of the different PTH forms. and the potencies were similar. This effect was already observed 15 min after injection. and was then close to or at its maximum (Fig. 4). (For calculation of % TRP. the Method section.)

Measurements of Cyclic Adenosine Monophosphate

The changes in the cAMP content of the urine after administration of PTH was somewhat variable. with the chemically synthesized hPTH showing the smallest effect. However. all forms of PTH responded in a similar fashion. Therefore. there is no principal difference between the preparations of PTH in terms of their stimulation of cAMP release into the urine (Fig. 5).

DISCUSSION

Structural analysis of PTH indicates that PTH(19–34) fragment contains substantial helical structure (17) and the residues 17–28 form an α-helix (15). This assumption has been confirmed (6). showing that mutations of the hydrophobic residues Leu²⁴, Leu²⁸. and Val³¹ in hPTH are critical for optimal PTH activity, in contrast to most mutations of the polar residues (i.e.. Lys²⁶, Lys²⁷. Gln²⁹. Asp³⁰. and His³²).

We have previously showed that QPTH is fully active in assays of adenylate cyclase. and this observation has been confirmed (6). Also in bone resorption studies using mouse calvaria (23), the QPTH was equally potent compared with the natural hormone. Biotinylation of Lys²⁶ or Lys²⁷ of [Nle⁸·¹⁸.Tyr³⁴]bPTH(1–34) has no effect on binding affinity (1). but substitutions as Lys²⁶ → Glu and Lys²⁶ → Thr causes partial reduction in cAMP production by PTH stimulation (6).

Interestingly. the substitution in QPTH. Lys²⁶ → Gln. lowers the hormone's affinity to the receptor 2.4 times. but does not influence the cAMP production compared to the wild-type hormone. indicating that the efficacy of the hormone receptor complex to stimulate the cyclase dependent G-protein(s) may still be similar. This certainly also shows that it is important to complement receptor binding studies with functional analysis.

Our in vivo studies have shown that the recombinant forms of hPTH are at least as potent as chemically synthesized hPTH

(29% higher doses of the chemically synthesized preparation were used), demonstrating that the yeast and *E. coli* hPTHs were correctly processed and that the molecule folded correctly to the proper tertiary structure, which is necessary to give full biological activity. Also, QPTH has folding characteristics that make it as active as the natural hormone. However, the reduced receptor binding potency and biological responses of the chemically synthesized hPTH on a molar basis is unexplained, but may be related to an inadequate *N*-terminal structure that is the last synthesized part of the peptide and for PTH is of crucial importance for receptor binding and eliciting the biological responses.

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less conducive to racemization than many other procedures, but probably even the best methods can cause racemization under adverse conditions.
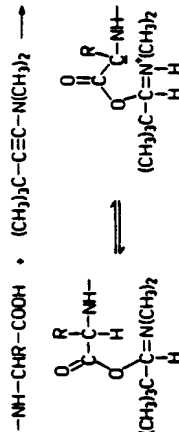
In the choice of coupling methods it is difficult to make positive recommendations, although some procedures, e.g. coupling via azides or with the help of EEDQ [52] have a fairly good record. It might be easier to point out coupling reagents which are notorious for their ability to cause racemization. Some of these, for instance the Woodward reagent [53], dicyclohexylcarbodiimide and other carbodiimides [54] caution the investigator by the structure of the reactive intermediates which contain a basic center, the potential cause of intramolecular proton abstraction from the chiral carbon atom:
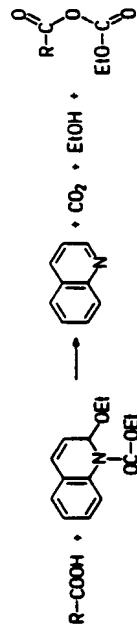
Similarly, among the various "push-pull acetylenes" [55–57] one with two basic centers [56]

is more conducive to racemization than others with only a single proton abstracting site. A basic center is generated in the earlier proposed [58] ynamines as well:
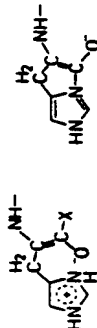
These considerations suggest that the lesser tendency of certain procedures to cause racemization is related to the absence of proton abstracting centers in the reactive intermediate and/or to the generation of materials which provide protons more readily than the chiral center of the activated residue. Thus, EEDQ [52] yields alcohol (and quinoline which has negligible basic strength):

In several coupling methods substances are released which are not acidic enough to prevent acylation of the amino component, but which can, nevertheless, effectively compete with the chiral center as proton donors. This is the situation with active esters which liberate substituted phenols or hydroxylamines during coupling.

In the base catalyzed racemization of reactive intermediates the amount and concentration of the base play an obvious role. The general principle to avoid basic conditions is supported by numerous reports and hardly requires further evidence. Thus, a free amine as nucleophile is preferable to a mixture of a salt of the amino component with a tertiary base. Weak acids, e.g. 1-hydroxybenzotriazole, do not interfere with acylation and coupling can be carried out without the addition of a tertiary amine [3]. Yet, over and above the *amount of the organic base* added to the reaction mixture its *chemical character* also has significant influence on the outcome of acylation. For instance, in mixed anhydride reactions, N-methylmorpholine causes less racemization [49] than the widely used triethylamine. In coupling via azides 1-diethylamino-2-propanol was found to be harmless [51] while triethylamine, N-methylmorpholine and diisopropylethylamine had, under certain conditions, an unfavorable effect on chiral purity. The last mentioned base prevents [39] the racemization of active esters of benzyloxycarbonyl-L-phenylglycine and of N-benzyloxycarbonyl-S-benzyl-L-cysteine, but had an almost as unfavorable effect on the optical purity of benzoyl-L-leucine p-nitrophenyl ester as other, less hindered, tertiary amines. Apparently steric hindrance in diisopropylethylamine is insufficient to interfere with proton abstraction from azlactone intermediates. Tribenzylamine seems to be more efficient in this respect. It is quite possible, however, that the influence of bases on racemization is determined not solely by their bulkiness but also by their basic strength [59, 60]. In this connection the racemization enhancing effect of the highly nucleophilic base p-dimethylaminopyridine [62, 63] should also be mentioned. On the other end of the scale, the weakly basic imidazole affects unfavorably the outcome of coupling reactions, particularly if its action is intramolecular. Thus, in acylation with activated derivatives of histidine significant racemization was observed [64], presumably caused by base catalyzed enolization or by cyclization and enolization.
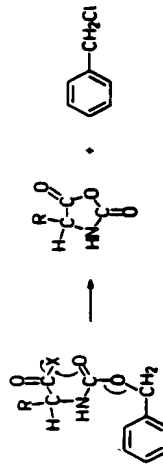
Substituents which reduce the basicity of the imidazole nucleus, e.g. the p-toluenesulfonyl group [65], reduce the extent of racemization as well [66]. Yet, a complete protection against loss of chiral purity of histidine residues can be expected only in derivatives in which the side chain protecting group (Y) is on the π-nitrogen atom of the imidazole:
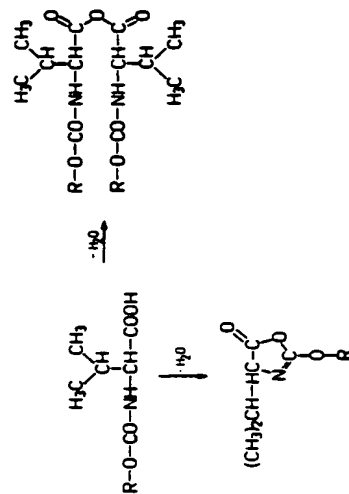
Among the factors which determine racemization the polarity of the *solvent* is quite important [4, 14]. In general, racemization is fast in highly polar solvents such as hexamethylphosphoramide, dimethylsulfoxide or dimethylformamide and is less pronounced in less polar solvents, e.g. pyridine, acetonitrile, chloroform, dichloromethane, tetrahydrofuran, dioxane or toluene. Unfortunately, most peptide intermediates are not sufficiently soluble in non-polar solvents and, at this time, the majority of acylation reactions are carried out in dimethylformamide. In solid phase peptide synthesis one applies solvents in which the peptidyl resin swells and a dissolution of the reactants is not needed. Thus, dichloromethane, which is not particularly conductive to racemization, can be used. An additional problem is created, however, by the solvent dependence of the rate of acylation of various activated intermediates. The most commonly used active esters react far better in polar solvents than in non-polar ones. These circumstances render the selection of solvents which would be favorable for acylation and yet cause little damage to chiral purity, rather difficult. A general remedy, which at least limits the extent of racemization, *is to carry out the coupling reactions at the highest possible concentration of the reactants* to ensure high coupling rates. This way the unimolecular, and hence concentration independent, racemization processes become less damaging.

A better approach to the conservation of chiral purity is offered by the *protecting groups* which are available for the blocking of the α-amino function. Already at the time of the introduction of the benzyloxycarbonyl group, its ability to protect against racemization during activation and coupling was noted and reported [67]. This unusual power to prevent the loss of chiral purity is absent from simple N-acyl groups such as the formyl, acetyl, trifluoroacetyl or benzoyl group and present only to some extent in the phthalyl group. On the other hand, several other amine protecting groups of the urethane type function equally well in this respect. Their ability to interfere with racemization was generally attributed to the lack of azlactone formation. The elimination of benzyl chloride and formation of N-carboxyanhydrides from Z-amino acid chlorides suggested [68] that
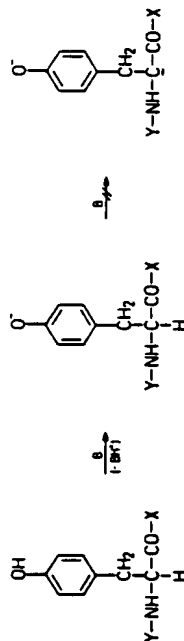

alkyloxycarbonylamino acids do not produce azlactones, the vulnerable intermediates. The formation of both the symmetrical anhydride and the 5(4H) oxazolone from benzyloxycarbonyl-L-valine and *tert*-butyloxy-carbonyl-L-valine on reaction with water soluble carbodiimides [15] demonstrates

the imperfectness of this rationale. It seems now that, while amino acids provided with a urethane-type amine protecting group do form azlactones, the latter retain their chiral integrity even under basic conditions. Thus, the former explanation requires revision, but the empirical rule that the benzyloxycarbonyl group and other urethane-type amine blocking groups prevent the racemization of the residues to which they are attached, remains valid. Notable exceptions are the blocked derivatives of S-alkyl-cysteine, O-alkylserine and β-cyanoalanine. Some other amine masking groups, e.g. the *p*-toluenesulfonyl and the *o*-nitrophenylsulfenyl group, are similarly protective in this respect.
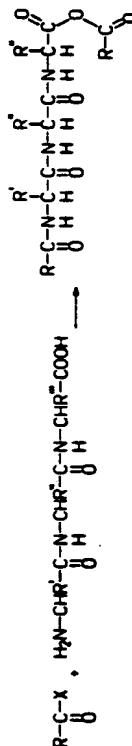
The *influence of the activated residue* on the extent of racemization can be considerable but it is not always fully understood. The benzylic character of the chiral carbon atom in phenylglycine offers a simple explanation. It is less easy to interpret the somewhat reduced chiral stability of phenylalanine moieties, probably caused by the electron withdrawing effect of the aromatic nucleus even if it is separated by a carbon atom from the chiral center. On the other hand, tyrosine with a free phenolic hydroxyl was not racemized [69] in the coupling of Z-Val-Tyr via its azide in the presence of excess base, while the azide of Z-Val-His suffered considerable loss in chiral purity under similar conditions. An explanation might be found in the abstraction of a proton from the phenolic hydroxyl: the resulting anion interferes with the abstraction of a second hydrogen and therefore the chiral carbon does not become an anionic center:

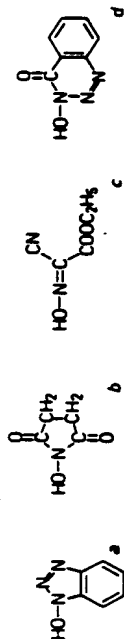Y—NH—C—CO—X →(B, i-BrY) Y—NH—C—CO—X →(B, ...) Y—NH—C—CO—X

In general, formation of dianions requires stronger bases than those used in peptide synthesis.

Racemization of activated valine and isoleucine residues occurs [70] in polar solvents. The electron release by the branched side chain should destabilize the anion which has to be assumed in base catalyzed racemization processes and thus an alternative rationale must be found. The known assistance of bulky substituents in cyclization reactions might contribute to the formation of cyclic intermediates, e.g. azlactones, which play a role in the process of racemization. It is equally possible, perhaps even more likely, that, because of steric hindrance caused by bulky side chains, the coupling reactions proceed rather slowly and this allows more time for the progress of racemization. Chiral integrity is affected also by the residue(s) which precede the activated C-terminal amino acid in a peptide and also by the bulkiness of the N-terminal amino acid in the amino component [70]. The sequence dependence of racemization received, so far, only limited attention [71] and clearly requires further systematic studies.

Racemization of the C-terminal residue of amino components with a free C-terminal carboxyl was an unexpected discovery [72]. This side reaction, which is enhanced by 1-hydroxybenzotriazole and suppressed by N-hydroxysuccinimide, is probably due to the transient activation of the unprotected carboxyl group through interaction with the acylating agent:

R—C—X , H₂N—CHR'—C—N—CHR''—C—N—CHR'''—COOH → R—C—N—C—C—N—C—C—N—C—N—CHR'''—COOH

One of the most powerful methods for the preservation of chiral integrity is the use of *additives* or, perhaps more appropriately, of *auxiliary nucleophiles*. These can reduce the lifetime of overactivated, racemization-prone intermediates, such as *O*-acyl-isoureas. Also, the commonly applied additives have acidic hydrogens and thus can provide a proton which is more readily abstracted by bases than the proton from a chiral center. The best results reported so far were achieved with 1-hydroxybenzotriazole [73] (a), *N*-hydroxysuccinimide [74, 75] (b), 2-hydroxyiminocyanoacetic acid ethyl ester [76] (c) and particularly with 3-hydroxy-3,4-dihydro-1,2,3-benzotriazine-4-one [77] (d).

(a) HO—N(benzotriazole)　(b) HO—N(succinimide, with CH₂—C=O groups)　(c) HO—N=C(CN)(COOC₂H₅)　(d) HO—N(benzotriazinone)
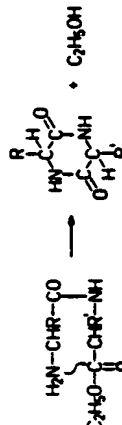
These racemization suppressing agents and several other potentially useful additives were compared by Izdebski [78].

From the foregoing discussion it is obvious that the extent of base catalyzed racemization is determined by a whole series of factors. An assessment of each of these in every coupling reaction is a demanding task and the results obtained so far are probably not entirely satisfactory since not all the influences are known, or at least not well enough to allow a quantitation of their contributions. Therefore, until the advent of truly racemization-free coupling methods, conservation of chiral integrity requires optimization in the choice of reagents, protecting groups, solvents, etc. Methods of activation which involve reactive intermediates containing a basic center should be used with caution. Overactivation, polar solvents should be avoided. The remaining choices are, however, not always conducive to an efficient formation of peptide bonds. Also, the selection of solvents is severely limited by the solubility of the intermediates. Hence, more weight has to be placed on the factors which provide some options and allow judicious decisions. For instance, the use of urethane-type amine protecting groups, attached to an amino acid rather than to a peptide, can greatly reduce the risk of racemization and the latter can be further diminished by avoiding the presence of tertiary bases in the reaction mixtures during activation and coupling. Last, but not least, the addition of well tested auxiliary nucleophiles creates conditions which no longer imperil chiral purity.

1.2 Undesired Cyclization

Dipeptide esters readily cyclize to form *diketopiperazines*. Ring closure can take place spontaneously because the thermodynamic stability of the six-membered ring overcomes the energy barrier in the formation of a *cis*-peptide bond, but the reaction is accelerated by bases, e.g. ammonia:

H₂N—CHR—CO / C₂H₅O—C—CHR'—NH → diketopiperazine + C₂H₅OH

In solid phase peptide synthesis [79], where frequently polymer bound benzyl esters are present, this side reaction can cause some premature cleavage of the chain from the insoluble support [80–83]:

benzyloxycarbonyl groups can be carried out by catalytic hydrogenation in the presence of organic bases [240]; under the same conditions, benzyl ethers are not cleaved [241]. Peptides which provide multiple ligands for palladium, e.g. compounds with more than one methionine residue, resist hydrogenation even in the presence of base. Forced conditions, e.g. catalytic reduction for prolonged periods of time, result in desulfurization and formation of α-aminobutyric acid residues [242]. Reduction with sodium in liquid ammonia remains a viable choice; but excess sodium demethylates the methionine side chain [243].

Oxidation of the thioether to a sulfoxide occurs during the operations of peptide synthesis or during purification, but can be prevented by working in an inert atmosphere. Fortunately, oxidation to the sulfoxide is reversible. A mild treatment with thiols will reduce a sulfoxide to the thioether. Sulfones cannot be reduced under mild conditions, but they also do not form from thioethers unless powerful oxidizing agents are used.

Alkylation of the sulfur atom in the methionine side chain readily occurs during the removal of blocking groups by acidolysis [111, 119]. Some alkylations are easily reversed; e.g. S-tert-butyl sulfonium salts decompose on standing or on warming with the regeneration of the thioether [121]. Alkylation by the benzyl group is a more serious side reaction because S-benzylmethionine (salts) give rise to a variety of products [244], among them S-benzylhomocysteine. Therefore, in reactions where alkylating agents are generated the thioether should be kept intact with the aid of scavengers. Alternatively the methionine side chain can be protected by oxidation to the sulfoxide [123] or by reversible alkylation with methyl p-toluenesulfonate [124]. Alkylation by chloromethyl groups of polymeric supports should be avoided.

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The
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of Peptides

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(25), NTA

Scheme 5.30.

Scheme 5.31.

case), and the progressive accumulation of over-reaction products limits the extent to which repetitive approach can be taken without difficult purification problems. The thio-analogues or NTAs (25) have also been used for peptide synthesis.[65] They are less prone to yield over-reaction products than NCAs, as the thiocarbamic acids produced by their aminolysis are less fragile. They are preferred in the special cases of glycine and histidine (the NCAs of which are especially subject to troublesome side-reactions), but unfortunately they are not secure against racemization.

5.1.2 Racemization*

Consider the synthesis of an all-L peptide comprising n chiral residues, from optically pure α-amino acids. If the operations needed for the incorporation of each residue result in conversion of a small fraction f of each residue to the D-form, and the epimers are carried through without separation, then the end product will consist of the required all-L peptide and a blend of other peptides in approximate proportions $(1-fn):fn$. For a synthesis of a 50-residue peptide in which 1% D-residue formation takes place at each stage, only half the final product will have the required all-L stereochemistry. The other 50% will consist mainly of about 1% each of all the 50 possible epimers with one D-residue. This will in general pose a prohibitive purification problem, and racemization in peptide synthesis has therefore been closely studied, with a view to defining the conditions under which it is minimal.[66-68]

Except for special cases (e.g., synthesis with N-methylamino acids: see Section 6.2.1), racemization is an almost exclusively base-induced side-reaction, and in practice is only a matter for serious concern at the activation and coupling stages of a synthesis. There are two important mechanisms.

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*This term is used in peptide chemistry in a loose way which not only covers the strict sense as defined in most general organic chemistry texts (conversion of an enantiomer to a mixture of enantiomers), but also embraces partial epimerisation, whereby there is loss of chiral integrity at one out of two or more chiral centres, resulting in the formation of a mixture of epimers (i.e. diastereoisomers differing at one chiral centre).

5.1.2.1 Direct enolization

Deprotonation at the α-carbon of an α-amino-acid residue results in racemization, because the carbanion intermediate can reprotonate on either side (Scheme 5.30). This has been called the 'direct exchange' mechanism, an inappropriate expression, because under some circumstances[69] racemization is much faster than exchange with the proton pool, implying that an ion pair is formed in which the ions are jostled about by solvent molecules and change their relative orientations without being divorced from each other, so that reprotonation can return the original proton to either side of the chiral centre ('isoracemization': Scheme 5.31). The rate of racemization by direct enolization depends on the catalysing base, the solvent, and the electron-withdrawing effects of the groups P,R, and X around the chiral centre (26). When X=NH-, O-alkyl, or O⁻, it is in most cases negligible,

P—NH—C(H)(R)—CO—X

(26)

and basic deprotection procedures other than saponification (see Section 4.1.1) are generally completely safe. During activation and coupling, the risk is rather more significant, but the danger is over once coupling is complete. Racemization is fastest with strongly electron-withdrawing groups X (i.e. most good leaving groups), and unhindered strong bases in dipolar aprotic solvents like DMSO and DMF. Inessential exposure to strong bases is clearly to be avoided, but the best way of responding to the other factors is not so obvious, because what matters is not the rate of racemization *per se*, but the balance between the rates of racemization and coupling, and this is much more difficult to make reliable generalizations about. Fortunately, with the exception of (a) a few special amino acids (α-arylglycines present quite a serious problem), and (b) couplings which are inordinately slow, the amount of racemization which actually takes place by this pathway is very slight indeed.

5.1.2.2 The oxazolone mechanism

Activated acylamino acids and peptides cyclize under the influence of base to give oxazolones [27; strictly '5(4H)-oxazolones', formerly '2-oxazolin-5-ones', archaically 'azlactones']. The oxazolones so formed are themselves activated towards aminolysis, and reaction with amino components leads ultimately to peptides, but since their racemization via stabilized anions is usually fast compared to the rate of peptide bond formation, any peptide thus produced is largely racemized (Scheme 5.32). Oxazolones are actually useful (e.g., references 70 and 71; see Scheme 6.31) for the activation of α-dialkyl-α-amino acid residues, where the question of racemization by base

Scheme 5.32. Conditions: basic.

(28)

Scheme 5.33.

does not arise because there is no hydrogen at the α-carbon. When the amino-nitrogen of the activated residue is acylated with a simple acyl group (acetyl, benzoyl, etc.), or with a peptide chain, cyclization to the oxazolone occurs easily with most good leaving groups X, and gross or even complete racemization may ensue. But oxazolone formation is not so facile when the acyl substituent is an alkoxycarbonyl protecting group. Indeed, the process was held to be impossible until 1977.[72] Furthermore, the alkoxyoxazolones 28 are both less easily racemized and more easily aminolysed than are the oxazolones 27 derived from simple acylamino acids. The activation of ordinary Z, Boc and Fmoc amino acids, etc., and their coupling with amino components is consequently not attended by the danger of racemization under normal conditions. This is a pivotal fact on which much of modern peptide synthesis turns. The reason for the contrast between, e.g., Z and benzoyl amino acids has not been fully explained, but a major factor is probably the lower acidity of BzlOCONH- compared to PhCONH-. In Scheme 5.32, the ring closure is shown as a specific base catalysed process, which has been demonstrated to be so in one set of circumstances.[72] It might be a concerted general base catalysed process under other conditions, but in either case lowering the acidity of the NH would be expected to diminish the rate of oxazolone formation.

A mechanism for oxazolone formation which does not require base assistance is also possible (Scheme 5.33), but ordinary amino acid derivatives do not cyclize this way except under very vigorous activation. N-Methyl-α-amino acid derivatives do, however, and give optically labile oxazolonium cations (see Scheme 6.29) even under normal activation conditions:[74] base catalysis is impossible because there is no NH for it to operate through, so cyclization can only occur by attack of the neutral amide oxygen on the activated carbonyl, which is easier with -CONMe- than -CONH- because

(29)

of electron release by the methyl group. Conformational restraints fortunately prevent this happening with proline derivatives, under all except the most extreme conditions.

Whether or not oxazolone-mediated racemization accompanies the activation and coupling of susceptible protected peptide acids depends on the leaving group. It does not seem to do so to a significant extent with acyl azide intermediates, in model systems at least, perhaps because these owe their aminolytic reactivity to intramolecular general base catalysis (see Section 5.1.1.2), to which oxazolone formation is indifferent because the nucleophile bears no hydrogen. And the risk is also small with the DCCI-HOBt procedure (see Section 5.1.1.4), because HOBt rapidly intercepts the activated species which might otherwise degenerate into oxazolones. The reactive HOBt ester intermediate favours aminolysis over oxazolone formation, possibly in part for the same reason as suggested for azides.

With activated protected peptides, the direct enolization and oxazolone mechanisms both provide pathways for the racemization of the carboxy-terminal residue. The formation of an oxazolone also threatens the chiral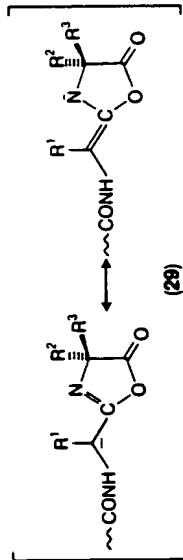 integrity of the penultimate residue, because the carbanion 29 is stabilised. Racemization at that residue has been observed at moderate levels in model experiments (e.g., reference 75), but has not so far been recognized as a real problem in actual syntheses, except when deliberate oxazolone formation is used to drive the coupling of carboxy components terminating in -XaaAibOH.[71]

5.2 The use of enzymes

This book is concerned with the chemical synthesis of peptides, so the inclusion of enzymic methods may raise a few eyebrows, but the use of enzymes as reagents in preparative organic chemistry,[76] without special homage to their biological origin, is burgeoning. A few remarks on enzymatic peptide bond formation[77,78] therefore seem called for.

Nature provides a wide range of proteolytic enzymes which can in principle be perverted to catalyse peptide bond formation by manipulating the conditions. There are two strategies for doing this. The first is dependent on thermodynamic control, the equilibrium in Scheme 5.34 (which

$$R'CO_2H + H_2NR^2 \rightleftharpoons R'CONHR^2 + H_2O$$

Scheme 5.34.

$$R'CO-X+H-Enz \longrightarrow R'CO-Enz \begin{array}{c} \nearrow H_2O \rightarrow R'CO_2H + H-Enz \\ \searrow R^2NH_2 \rightarrow R'CONHR^2 + H-Enz \end{array}$$

Scheme 5.35.

ZAspOH+HPheOMe $\xrightarrow{i}$ ZAspPheOMe $\xrightarrow{ii}$ HAspPheOMe

(30)
Aspartame

Scheme 5.36. Conditions: i, 2 equiv. HPheOMe/pH7/thermolysin (the dipeptide salt precipitates); ii, HCl, then catalytic transfer hydrogenolysis with $HCO_2NH_4/Pd(C)/MeOH$.

favours hydrolysis overwhelmingly under normal conditions) being somehow displaced in favour of peptide bond formation. This can be achieved by employing protecting groups which will ensure precipitation of the peptide, or by using biphasic systems so that the peptide passes out of the aqueous phase into an organic solvent as it is formed, or by using water-miscible organic solvents which perturb the dissociation constants of the components and shift the balance of the equilibrium. The second strategy exerts kinetic control by arranging for an amino component nucleophile to compete with water for an acyl-enzyme intermediate (Scheme 5.35). The advantages of an enzymatic synthesis are the mild conditions, freedom from racemization and the need for side-chain protection, the possibility of using immobilized enzyme technology[79,80] with catalyst recovery, and the scope for industrial scale-up. Many examples have been reported. The synthesis[81] of the synthetic sweetener aspartame (30) is one of particular interest which has been developed for commercial application, and is also simple enough to be an undergraduate exercise[82] (Scheme 5.36). There are disadvantages, however. With peptides longer than dipeptides, there is the danger that while the protease is being persuaded to work backwards in creating a peptide bond at one point, it will remember the purpose for which evolution devised it and dismantle another somewhere else. No new case can be treated as

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Recombinant DNA

A Short Course

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Open Reading Frames in DNA Delineate Protein-Coding Regions

A computer can also be used to analyze a long DNA sequence to determine the location of regions that may code for proteins. The computer is instructed to search for "open reading frames," long stretches of triplet codons that are not interrupted by a translational stop codon. This procedure can be very useful when a cloned DNA fragment is known from, say, some functional assay to contain a certain gene, but when the size of the gene or its location on the fragment is not known. If an open reading frame can be found somewhere in the sequence—especially if the frame has an ATG (the universal translation-initiation codon) near the start—it is very likely that this stretch of sequence is in fact the gene; discovery of an open reading frame does not *prove* the existence of a gene, of course, but it at least delineates an area to home in on. Conversely, the lack of an open reading frame in a stretch of sequence that was thought to contain a gene has been used to determine that some "genes"—chromosomal sequences that hybridize to specific mRNAs—are in fact pseudogenes, nonfunctional relics that arose during the evolution of gene families. Computer searches for open reading frames have even pointed out sequences that code for mRNAs (and probably proteins) that were previously unsuspected. The long terminal repeat (LTR) of mouse mammary tumor virus (Chapter 10) and a stretch of adenovirus DNA, for example, were found to have long open reading frames that have since been found to code for mRNAs. The proteins coded for by these mRNAs have not yet been determined, but no one would have even *looked* for the mRNAs if the open reading frame had not been found.

Leader Sequences at the NH$_2$-Terminal Ends of Secretory Proteins

DNA sequence analysis reveals that many functional proteins first exist in the form of slightly larger precursors containing some 15 to 25 additional amino acids at their NH$_2$-terminal ends. Such "leader" (signal) sequences are diagnostic of proteins that move through cellular membranes to function only after they have been secreted from the cells in which they were made (examples of such proteins are insulin, serum albumin, antibodies, and digestive tract enzymes), or after they have been anchored to the outer surface of a cell membrane (the histocompatibility antigens on the cell surface are an example). A majority of the amino acids found in leaders are hydrophobic, and they somehow function to ensure both the attachment of nascent polypeptide chains to appropriate
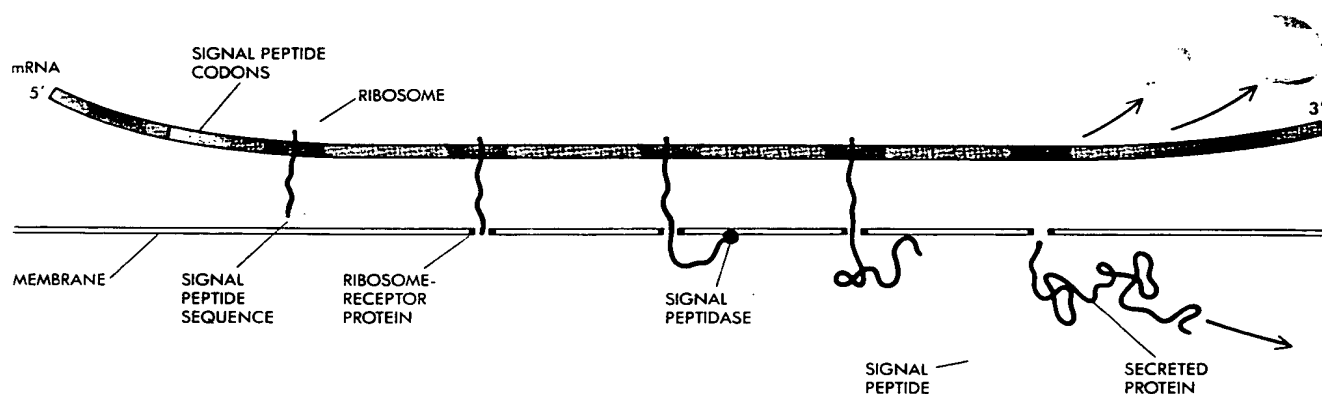
Figure 7-6
Signal sequences. Proteins destined to be secreted from the cell have an N-terminal sequence that is rich in hydrophobic residues. This "signal" sequence binds to the membrane and draws the remainder of the protein through the lipid bilayer. The signal sequence is cleaved off of the protein during this process by an enzyme called signal peptidase.
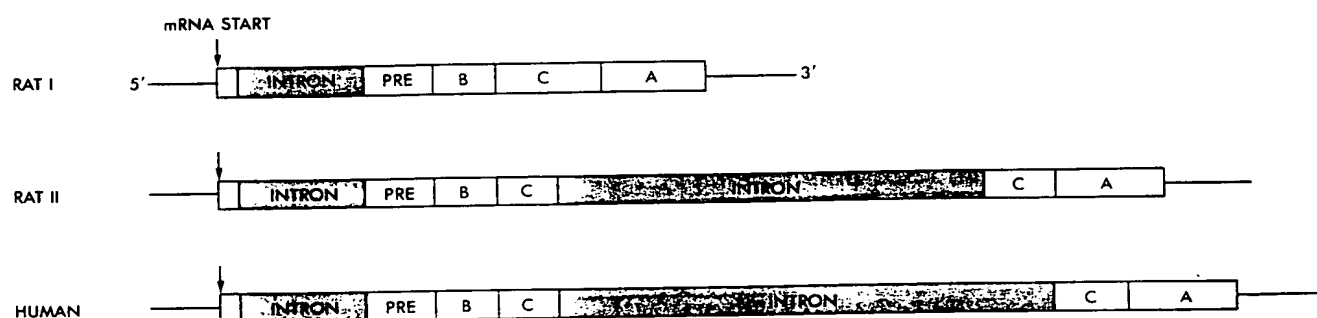
Figure 7-7
A comparison of rat and human insulin genes. Pre, A, B, and C represent the different peptide domains of the proinsulin molecule.

membranes, and the subsequent passage of the chains across the lipid bilayers that characterize all cellular membranes. *In vivo,* leader sequences usually have only a fleeting existence, because they are cleaved off by specific proteolytic enzymes that generate the NH_2-terminal amino acids of the functional secreted products (Figure 7-6).

Introns Sometimes Mark Functional Protein Domains

At first, neither the location nor the number of introns within a given gene made sense. In rats, for example, two closely related genes code for insulin—one gene has only one intron and the other has two. The rat insulin I and rat insulin II genes have introns of almost identical sizes located immediately downstream from the sequences coding for the insulin leader. The second intron of the rat insulin II gene is located within the so-called "C" segment of the insulin protein precursor that is digested away to produce the two-chained structure of mature insulin molecules. Humans have only one insulin gene whose two introns are located in positions similar to those of the rat insulin II gene (Figure 7-7), thus suggesting the descent of rat and human genes from a common ancestor. No obvious functional difference marks the amino acids separated by the second insulin intron, whose location might be accidental.

In hemoglobin, though, the amino acids constituting the special functional domain surrounding the heme group are clearly delineated by an intron from the more distal amino acids. As we describe below, introns within antibody genes are precisely located between functional domains. For this reason, much protein evolution may have been accomplished by genetic recombination events that brought together domains previously located on separate genes. It is conceivable that the long length of many introns helps to ensure that coding sequences are kept intact during genetic crossing over.

Alternative Splicing Pathways Generate Different mRNAs from a Single Gene

RNA splicing can also generate different mRNAs and thus different proteins from one gene, or, more accurately, from one primary transcriptional unit. Differential splicing was first seen in the adenoviruses and then in SV40, in polyoma virus, and in the mRNAs coding for immunoglobulins. A recent example involves the mRNA coding for the hormone calcitonin, a peptide that is normally produced in large amounts in the thyroid gland. Although a large amount of calcitonin mRNA is present in the hypothalamus, very little calcitonin itself is produced there. Instead, another protein that is called "calcitonin-gene-related product" or CGRP, and whose function is still unknown, has been detected. Both calcitonin and CGRP are produced from the same primary transcript by using alternative splicing routes. The routes used produce two different mature mRNAs having a common 5' end but different 3' ends: The thyroid

early region have been produced in this way (Figure 15-2b). The advantage of replacing early genes by foreign DNA and propagating virions in COS cells is that *all* progeny virus particles should have the recombinant genome; there are no contaminating helper viruses.

Analysis of Cloned Surface Antigen Genes

A gene inserted into an SV40 vector and introduced into animal cells should be efficiently and correctly expressed. The protein that is made should be fully functional; it should undergo any posttranslational modification that it normally undergoes, and, if it is a protein that is ordinarily transported through the intracellular membrane system to the cell surface, this transport process should be completed. Cloning in SV40 of a gene coding for such a protein provides an opportunity not only to study transcription, mRNA splicing, and translation, but also the intracellular transport mechanisms that sort and deliver the protein to its correct cellular location, and the mechanisms that insert and anchor it in membranes. Through the use of *in vitro* mutagenesis (Chapter 8), amino acid codons in the gene can be selectively mutated and the effects of such mutations can be assessed. In this way it should be possible to define the functional domains of the gene and its corresponding protein.

For example, the flu virus hemagglutinin (Ha) gene cloned into SV40 expression vectors is efficiently expressed in infected monkey cells (Figure 15-3). The hemagglutinin is fully glycosylated and transported in a normal way to the cell surface membrane. There it remains in a biologically and antigenically active form, anchored through a carboxyl-terminal domain containing hydrophobic amino acids that lie in the lipid bilayer of the membrane.

When cells were infected with the SV40 vector carrying an Ha gene from which the sequences that specify the carboxyl-terminal hydrophobic segment had been deleted, the hemagglutinin was synthesized and glycosylated, but was then secreted into the culture medium rather than being anchored to the membrane. This neatly and unambiguously proved that the C-terminal hydrophobic domain of the hemagglutinin is responsible for
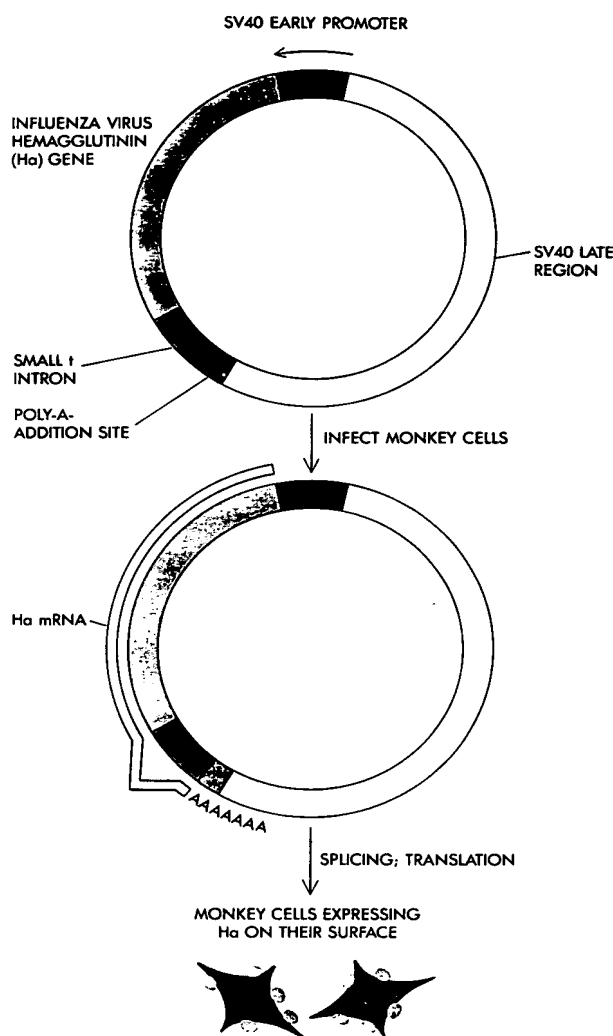
Figure 15-3
The SV40 early promoter can be used to express large amounts of flu virus hemagglutinin.

maintaining it as an integral membrane protein. As we have mentioned (Chapter 7), the amino-terminal segment of both secreted and integrated outer-surface membrane proteins in eukaryotic cells is also hydrophobic, and acts as a leader to translocate the nascent polypeptide across the membranes of the intracellular transport system. During this translocation, the amino-terminal leader is cleaved from the growing polypeptide. Removal of the 5' end of the cloned Ha gene, which codes for the

hemagglutinin leader, resulted in hemagglutinin that was not glycosylated and was not translocated across intracellular membranes.

Experiments such as these would not be possible without eukaryotic cloning vectors. Cloning in bacteria would allow study of the early steps in gene expression, but not of posttranslational modifications such as glycosylation and transport through intracellular membrane systems. How proteins are sorted within cells and delivered to their correct destinations is now a central preoccupation of cell biologists. Gene cloning provides a way of tackling the problem.

Plasmidlike Replication of DNA in COS Cells

The SV40 vectors we have just discussed depend on the completion of the replication cycle of the virus. In COS cells, *any* piece of DNA that includes an SV40 origin of replication will replicate because of the presence of SV40 T antigen in the cells. The foreign DNA will, at least transiently, replicate independently of the cellular DNA.

Recombinant DNAs containing the SV40 origin and a foreign gene will, when introduced into COS cells, replicate to an enormously high copy number. The transcription of the foreign gene from the plasmidlike DNA molecules can then be studied. In one such experiment, transcription of the β-globin gene was found to initiate correctly and to produce a precisely spliced mRNA (Figure 15-4). Because so many gene copies are present in each cell and because the total amount of RNA made is correspondingly high, this system provides a quick way of screening mutations for their effects on transcription and RNA processing.

Rescue of Integrated SV40 DNA by COS Cell Fusion

Nonpermissive mouse cells transfected with a plasmid containing the SV40 origin of replication linked to some selectable marker will incorporate the DNA into the chromosomes. If these cells are then fused with COS cells (fusion can be effected with polyethyleneglycol), the T antigen being produced by the COS cells will diffuse to the nuclei of the mouse cells and initiate DNA replication at the SV40 origins in the mouse cell chromosomes.
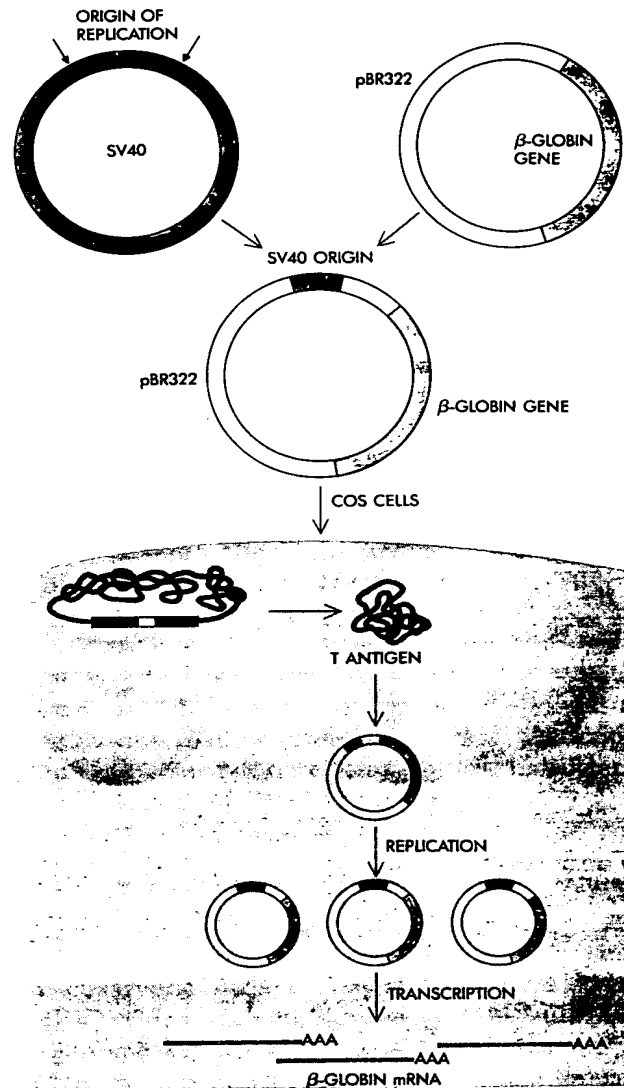
Figure 15-4
A foreign gene cloned on a plasmid with the SV40 origin replicates to a very high copy number when transfected into COS cells. The high copy number allows efficient transcription of the foreign gene.

The replication proceeds bidirectionally and eventually produces circular molecules that pop out of the chromosomes. These molecules can easily be purified away from the bulk of the chromosomal DNA. If plasmid pBR322 sequences are located near the SV40 origin, the circular molecules produced in this manner can be propagated directly in

United States Patent [19]

Brewer et al.

[54] **HUMAN PARATHYROID HORMONE**

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[22] Filed: **Dec. 10, 1973**

[21] Appl. No.: **423,303**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 317,702, Dec. 21,
1972, abandoned.

[52] U.S. Cl.............................. 260/112.5; 424/177
[51] Int. Cl.. C07c 103/52; C07g 7/00; A61k 27/00

CCuS 530/324, 399

[58] Field of Search.................... 260/112.5; 424/177

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3585–3588 (1972).

Primary Examiner—Lewis Gotts
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[57] **ABSTRACT**

Human parathyroid hormone was isolated in highly
purified form from human parathyroid adenomas. The
primary sequence of the amino terminal 34 residues
was determined and the peptide of the first 34 resi-
dues synthesized.

1 Claim, 2 Drawing Figures

FIG. 1

AMINO ACID SEQUENCE OF THE AMINO TERMINAL 34 RESIDUES OF HUMAN PARATHYROID HORMONE
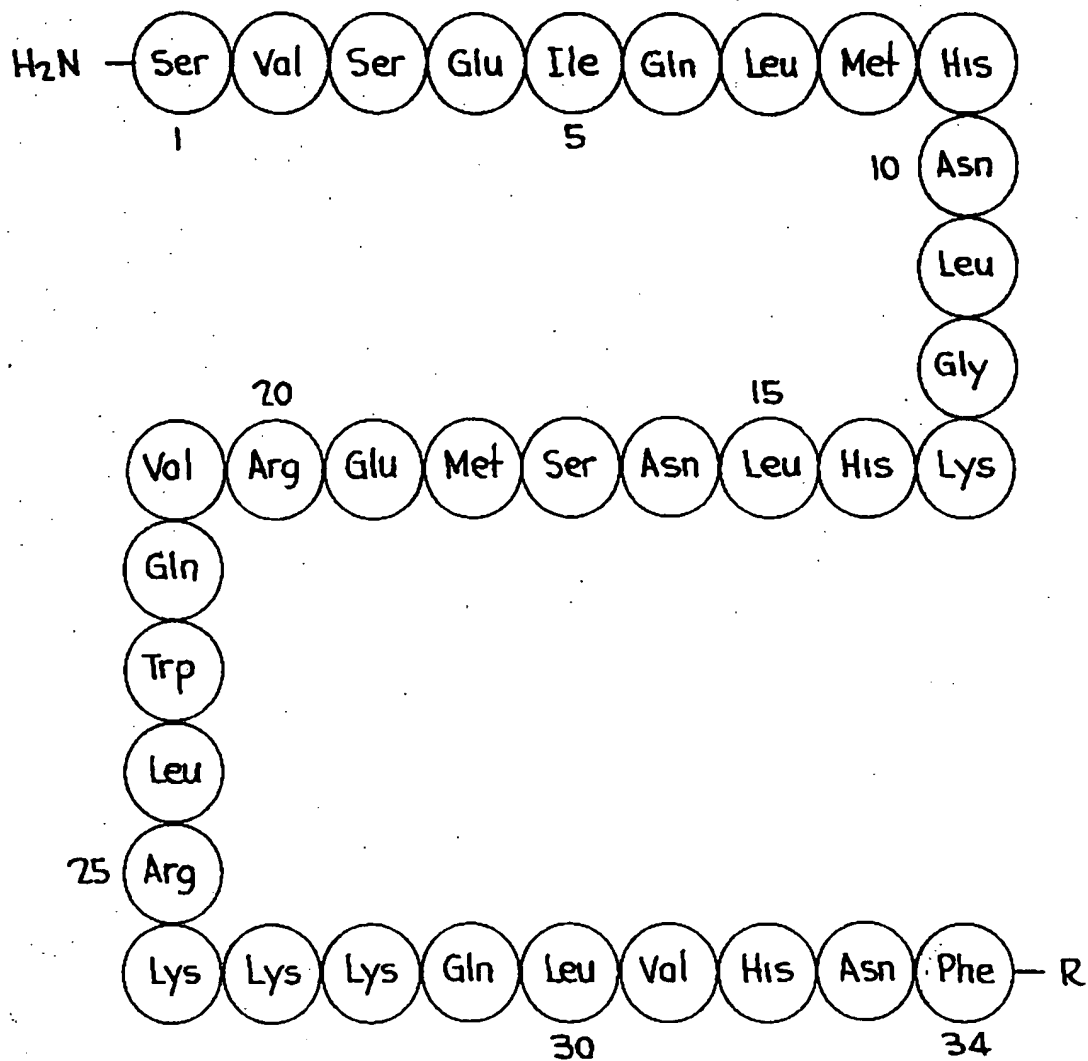
FIG.2

HUMAN PARATHYROID HORMONE

H₂N —(Ser)(Val)(Ser)(Glu)(Ile)(Gln)(Leu)(Met)(His)(Asn)(Leu)(Gly)(Lys)(His)(Leu)(Asn)(Ser)(Met)(Glu)(Arg)(Val)(Glu)(Trp)(Leu)(Arg)(Lys)(Lys)(Gln)(Leu)(Leu)(Val)(His)(Asn)(Phe)—R

1   5   10   15   20   25   30   34

BOVINE PARATHYROID HORMONE

H₂N —[Ala](Val)(Ser)(Glu)(Ile)(Gln)[Phe](Met)(His)(Asn)(Leu)(Gly)(Lys)(His)(Leu)[Ser](Ser)(Met)[Glu](Arg)(Val)[Glu](Trp)(Leu)(Arg)(Lys)(Lys)[Leu](Gln)[Asp](Val)(His)(Asn)(Phe)—R

1   5   10   15   20   25   30   34

PORCINE PARATHYROID HORMONE

H₂N —(Ser)(Val)(Ser)(Glu)(Ile)(Gln)(Leu)(Met)(His)(Asn)(Leu)(Gly)(Lys)(His)(Leu)⟨Ser⟩(Ser)⟨Leu⟩(Glu)(Arg)(Val)⟨Glu⟩(Trp)(Leu)(Arg)(Lys)(Lys)⟨Leu⟩(Gln)⟨Asp⟩(Val)(His)(Asn)(Phe)—R

1   5   10   15   20   25   30   34

1
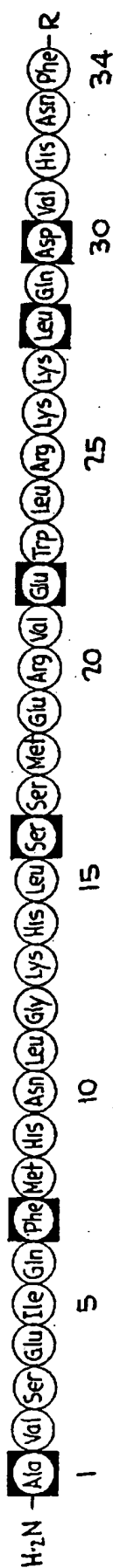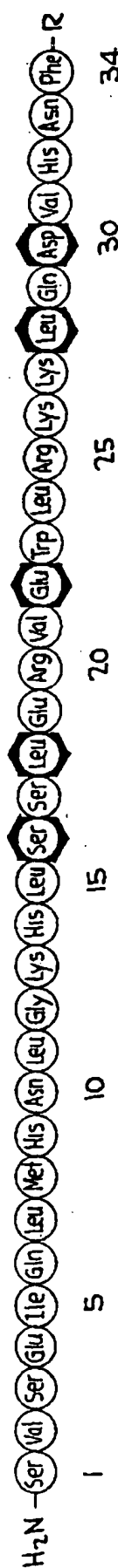
HUMAN PARATHYROID HORMONE

This application is a continuation-in-part of copending application Ser. No. 317,702, filed Dec. 21, 1972, now abandoned.

This invention relates to peptides, and more specifically to the biologically active amino terminal 34 residues of the human parathyroid hormone.

During the last few years a significant core of information has been obtained by a number of laboratories on the chemistry, biosynthesis, and secretion of the parathyroid hormone (PTH). These studies have indicated that the parathyroid hormone is initially synthesized as a prohormone, proparathyroid hormone. Proparathyroid hormone contains approximately 106 amino acids, and has an apparent molecular weight of 12,500. The prohormone is rapidly converted into the storage or glandular form of the hormone consisting of 84 amino acids, and a molecular weight of 9,500. The complete amino acid sequences of the 84 amino acid parathyroid hormone from the bovine and porcine species have been reported. Following appropriate physiological stimuli the 9,500 molecular weight form of the parathyroid hormone is secreted into the circultion. Shortly after entering the peripheral circulation the glandular form of the hormone is cleaved into smaller fragments. Gel filtration of human hyperparathyroid serum by several investigators have revealed a major immunoreactive fragment(s) with a molecular weight of 5–8,000 and several minor components. Immuno-chemical heterogeneity of the circulating human para-thyroid hormone, presumably due to the different molecular forms of PTH, was initially reported by Berson and Yalow (*J. Clin. Endo. Met.* 18, 1037–1047 (1968)), and has been confirmed by others. The specific site(s) of cleavage in the 84 amino acid polypeptide chain of the parathyroid hormone in the general circulation is as yet unknown, and the biological activity of the resulting fragments which make up the majority of the immunochemical circulating hormone has not been reported. A biologically active peptide fragment of bovine PTH, prepared by dilute acid cleavage, has been reported, indicating that the intact 84 amino acid polypeptide is not needed for biological activity. This peptide has been identified as the amino terminal peptide of the hormone, and is composed of the initial 30 residues of the sequence (Keutmann et al, *Biochem.* 11, 1973–1979 (1972)). Synthetic peptides of the first 34 residues of the bovine hormone, and the initial 30 residues of the procine hormone have been prepared and are biologically active, thereby confirming the localization of the biologically active region of the parathyroid hormone to the amino terminal third of the 84 amino acid polypeptide chain. There has, heretofore, been no identification of the sequence of the initial residues in the human hormone. It follows, however, that the biological activity of the human hormone would lie in the first 34 residues.

It is, therefore, a primary object of the present invention to obtain the primary sequence of the amino terminal 34 residues of human PTH.

It is another object of the present invention to provide a synthetic peptide comprising the amino terminal 34 residues of human PTH.

ISOLATION AND IDENTIFICATION

The human parathyroid hormone used in these stud-

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ies was isolated from parathyroid adenomas obtained from patients undergoing surgery for hyperparathy-roidism. Dried, defatted parathyroid tissue was initially extracted with 8M urea in 0.2N hydrochloric acid, and fractionated with ether, acetic acid, sodium chloride, and trichloroacetic acid (TCA powder) according to the procedure of Rasmussen et al in *J. Biol. Chem.* 239, 2852–2857 (1964). The TCA powder was further purified by gel filtration, followed by ion exchange chromatography on CM-sephadex employing an ammonium acetate gradient. The isolation of the hormone was monitored by radioimmunoassay and disc gel electro-phoresis.

Amino acid analyses were performed on a Beckman-Spinco automatic amino acid analyzer, Model 120B or 121 adapted for high sensitivity or a Durrum Model 500 analyzer. Analytical disc gel electrophoresis was performed in 8M urea at pH 4.4 as previously reported by Brewer et al in *J. Biol. Chem.* 246, 5739–5742 (1970). Immunoassays were performed by the procue-dure of Arnaud et al in *J. Clin. Invest.* 50, 21–34 (1971).

Automated Edman degradations were performed with the Beckman Sequencer, Model 890B, utilizing a 1M Quadrol buffer. The phenylthiohydantoin (PTH) amino acids were identified by regeneration to the constituent amino acid by hydrolysis with hydroiodic acid for 20 hours at 130°C., 1 gas liquid chromatography (2,3) and mass spectrometry. (4,5,6) chemical ioniza-tion (CI) mass spectrometry was performed on a Finni-gin mass spectrometer equipped with a PDP-8/e Digital computer, and a Complot Plotter. Isobutane was used as the carrier gas, and the source was maintained at 200°C. The samples were applied by a direct inser-tion probe, and the probe was heated from 30° to 250°C. over a 90 sec period. Electron impact (EI) mass spectrometry was performed on an LKB mass spec-trometer Model 9000 using a direct insertion probe and an electron energy of 70eV.

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The purified human parathyroid hormone migrated as a single component on disc gel electrophoresis with a mobility which was identical to that of the bovine pa-rathyroid hormone. Amino terminal analysis of the pu-rified peptide by the Edman technique revealed serine.

350 nanomoles of the purified hormone were de-graded on the Beckman Sequencer using a single cleav-age of heptofluorobutyric acid at each degradation. The results of the degradation of the first 34 residues of the human parathyroid hormone are shown in FIG. 1. A "quasimolecular" (QM+) or major fragmentary ion is observed in each of the CI mass spectra. At step 12 in the sequence a quasimolecular ion for glycine (m/e 192) and leucine (m/e 249) are observed. Quanti-tation by the gas chromatography method of glycine (.28 μM) and leucine (.09 μM) permits definite identi-fication of glycine as the twelfth amino acid in the se-quence with the leucine resulting from overlap from step 11 (FIG. 1.). Leucine/isoleucine and lysine/gluta-

mine yield identical masses of m/e 264 respectively on CI mass spectrometry. Lysine, however, can be distinguished from glutamine by the fragmentary ion at m/e 306. Lysine/glutamine and leucine/isoleucine were also readily differentiated by gas chromatography on the CFC blend and by EI mass spectrometry.

These combined results provided a single unique sequence for the first 34 residues of the human parathyroid hormone (FIG. 1)

UTILITY

The amino acid sequence of the first 34 residues of human parathyroid hormone is of major importance since previous studies of the bovine and procine species have indicated that this is the biologically active region of the native hormone. The first 34 residues of human PTH differ from the bovine by 6 residues, and the porcine by 5 residues (FIG. 2). The amino terminal 15 residues of human and procine PTH are identical, however bovine differs from human and porcine PTH in positions 1 and 7 where alanine substitutes for serine, and leucine replaces phenylalanine (FIG. 2). In the remaining 16–34 region human PTH differs from porcine PTH by 5 residues, and from bovine PTH by 4 residues (FIG. 2). Human PTH contains 2 methionine residues similar to the bovine species, whereas porcine PTH contains a single methionine at position 8 (FIG. 2). The human sequence is unusual in that it contains 4 consecutive basic residues (arginine residue 25, and lysine residues 26 to 28). Amino acid residues in the first 34 which are unique to the human sequence include an asparagine at position 16, glutamine at position 22, lysine at position 28, and a leucine at position 30.

One of the major problems in the clinical assessment of patients with disorders of mineral metabolism has been the difficulties encountered with the radioimmunoassay of human parathyroid hormone. There have been two basic problems with the immunoassay of PTH. The first problem, as discussed earlier, has been the presence in the peripheral circulation of peptide fragments of the 84 amino acid polypeptide chain. The antisera from various laboratories undoubtedly have immunological determinants for different regions of the intact molecule, thus leading to variable and sometimes inconsistent results when applied to the measurement of PTH circulation in human blood. In addition, the differentiation by immunoassay of biologically active amino terminal fragments from inactive fragments has as yet been impossible. The second difficulty has been the utilization of heterologous assays employing radioactive labeled bovine hormone as the tracer, and antibodies prepared against the bovine or porcine hormone. The sensitivities of these assays are therefore variable, and depend on the cross reactivity of the particular antiserum with the human hormone. As noted above, the human sequence in only the initial third of the molecule differs from the bovine by 6 residues and the porcine by 5 amino acids.

Habener, et al (*Nature New Biology* 238, 152–154 (1972) have attempted to circumvent some of these problems with the immunoassay by the development of amino and carboxyl specific antisera. These investigators have used an antibody prepared against the bovine hormone, and have absorbed their antiserum with either the synthetic 1–34 bovine fragment, or a 53–84 fragment prepared by chemical cleavage of the native bovine hormone. The amino terminal specific antise-

rum was further characterized by displacement with synthetic bovine fragments, and the recognition site of this absorbed antiserum was shown to be directed toward residues 14 to 19 in the bovine sequence. Using this approach, they have concluded that the major fragment in the human circulation is carboxyl terminal, and biologically inactive. They were, however, unable to identify the amino terminal fragment in the circulation of human subjects. This may be due to either the rapid clearance of the amino terminal specific bovine antiserum with the amino terminal region of the human hormone. It is of interest that the human sequence differs in the 14 to 19 region from the bovine hormone by the substitution at step 16 of an asparagine for a serine residue (FIG. 2). The significance of this substitution in the human hormone to the results they have obtined with their amino terminal specific bovine antiserum is as yet unknown. Canterbury and Reiss have reported results on the nature of the circulating fragment of the parathyroid hormone that are in contrast to those reported by Habener, et al. Using an antiserum prepared against bovine parathyroid hormonnc, these investigators have identified three different immunochemical forms of the parathyroid hormone in the peripheral circulation of hyperparathyroid patients (*J. Clin. Invest.* (in press) (1973)). The molecular weight of these three components, as determined by gel filtration, were 9500 (presumably glandular PTH), 7000–7500, and 4500–5000. Recently these investigators have directly accessed the biological activity of these three fragments in a renal adenyl cyclase system. Both the 9500 and the 4500–5000 fragment stimulated the adenyl cyclase system, whereas the 7000–75000 component was inactive. These results are consistent with the presence of an amino terminal active fragment of PTH of approximately one-half the size of the glandular hormone in human hyperparathyroid serum.

The determination of the amino terminal sequence of the human parathyroid hormone now permits the synthesis of peptides based on the human sequence for both clinical and investigative use. Synthetic fragments, as well as chemical analogues, permit more definitive studies to be performed on the chemistry of the human hormone, including the specific residues and the minimum length of the polypeptide chain that is required for biological activity. In addition, these synthetic fragments enable investigators to characterize the heterologous antisera currently in use in the immunoassay, and to develop specific antisera directed toward the amino terminal region of the human hormone. Antisera based on the human sequence will enable more detailed studies to be performed on the nature of the circulating hormone in man, and its role in calcium homeostasis and metabolic bone disease.

Clinically, the synthetic hormone can be used for replacement therapy for the natural human parathyroid hormone. The peptide is administered in microgram quantities by intravenous (IV) or intramuscular (IM) injection. Actual dosage is dependent on many factors including, but not limited to, the patient's tolerance, side effects, and the like, but can be routinely determined by one of ordinary skill in the art. The vehicle for the hormone would be any physiologically tolerable vehicle having approximately neutral pH, such as physiological saline solution. The synthetic hormone can also be used in diagnostic procedures, based on the fact that parathyroid hormone produces hypercalcemia, hy-

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pocalcemia, hyperphosphaturia, and increased urinary cyclic AMP in normal individuals. In this procedure the patient's response is evaluated by administering the peptide either IV or IM and the serum calcium, urinary calcium, phosphate, and cyclic AMP is monitored.

SYNTHESIS

The peptides based on the human sequence are synthesized by either of two known methods. The first is the solid phase synthesis technique of R. B. Merrifield et al. reported in *Advances in Enzymology* 32, 221 (1969) and forming the subject matter of U.S. Pat. No. 3,531,258 issued on Sept. 29, 1970, the subject matter of which is incorporated herein by reference. The second is the classical synthesis described by M. Bodanszky and M. A. Ondetti in *Peptide Synthesis*, Interscience (New York 1966) the subject matter of which is incorporated herein by reference.

THE SOLID PHASE SYNTHESIS

The solid phase method of synthesizing a peptide chain, according to Merrifield et al, is based on the fact that the chain can be synthesized in a stepwise manner while one end of the chain is covalently attached to an insoluble solid support. During the intermediate synthetic stages the peptide remains in the solid phase and can therefore be manipulated conveniently without significant losses.

The automation of the process carried on by the apparatus of Merrifield et al is possible because all of the reactions, including the intermediate purification procedures, are conducted within a single reaction vessel. The apparatus also solves the problem of introducing the proper reagents and solvents into the vessel in the proper sequence at the proper times while maintaining sufficient flexibility to cope with a wide range of reactions and conditions which may occur due to modification of each of the reactions in the synthesis.

During the process the solid support is a chloromethylated styrene-divinylbenzene copolymer bead. The C-terminal amino acid is coupled as a benzyl ester to the resin and the peptide chain grows one residue at a time by condensation at the amino end with N-acylated amino acids. the tert-butyloxycarbonyl group has been the protecting group of choice and activation has usually been by the carbo-diimide or active ester routes.

In general in the apparatus of Merrifield et al, the proper reagents and solvents are selected by the solvent and the amino acid selector valves and are transferred by the metering pump from one of the reservoirs to the reaction vessel which contains the peptide-resin. After the desired period of mixing by the shaker the solvents, excess reagents and by-products are removed to the waste flask by vacuum filtration. These basic operations are repeated in prearranged sequence under electrical control until the synthesis of the desired peptide chain is complete. All parts of the apparatus which come into contact with solvents and reagents are made of glass or chemically resistant polymers.

Several preliminary operations are necessary before the synthesis of a peptide can be started. First, the supporting resin containing the C-terminal amino acid of the proposed peptide chain must be prepared and analyzed. This is done by esterification of a chloromethylated copolymer of styrene and divinylbenzene with the tert-butyloxycarbonyl (t.—BOC) amino acid. The product is freed of very fine particles of resin by flota-

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tion in methylene chloride to prevent subsequent clogging of the fritted discs of the reaction vessel. A sample of the vacuum-dried product is hydrolyzed in a 1:1 mixture of dioxane and 12 N HCl and the liberated amino acid is measured quantitively on an amino acid analyzer. The amino acid content is used to calculate the amounts of subsequent amino acid derivatives and dicyclohexylcarbodiimide reagent which will be used in the synthesis. The best range of substitution has been 0.1 to 0.3 mm. per gram. The tert-butyloxylcarbonyl amino acidresins are usually prepared in advance and are stored until needed.

The appropriate solvent reservoirs are filled with glacial acetic acid, methylene chloride and commercial (99.5%) absolute ethanol. N-N-Dimethylformamide is freed of dimethylamine and formic acid by shaking with barium oxide and distillation under reduced pressure. The 1 N HCl-acetic acid solution is prepared by adding 700 ml. of glacial acetic acid to the storage separatory funnel and passing in a slow stream of anhydrous hydrogen chloride. Samples are withdrawn at the bottom and titrated for chloride by the Volhard method. This solution, when protected by the long coil of capillary tubing and drying tube, is stable for several weeks without a significant decrease in concentration. The triethylamine reagent is prepared by mixing 50 ml. of triethylamine with 450 ml. of purified dimethylformamide.

The reaction vessel is loaded with a weighed amount of the t-BOC amino acid-resin (2 to 4 grams for a small, 45 ml. capacity vessel). The stopper is lubricated with silicone high vacuum grease and secured in place with springs, and the inlet and outlet lines are attached. In the synthesis three equivalents of each T.—BOC amino acid derivative are used per equivalent of the first amino acid on the resin. The calculated quantity of each of the first six amino acids is dissolved in 7 ml. of methylene chloride, filtered if necessary, and placed in the amino acid reservoirs in the proper sequence. Because of poor solubility in methylene chloride, t.—BOC-nitro-L-arginine is first dissolved in 2 ml. of dimethylformamide and diluted with 5 ml. of methylene chloride, while t.BOC-im-benzyl-L-histidine is dissolved in 7 ml. of pure dimethylformamide. The t.—BOC amino acid-p-nitrophenyl esters are dissolved in 16 ml. of pure dimethylformamide. During the automated synthesis the amino acid solutions are pumped completely into the reaction vessel and a precise concentration therefore is not required. The dicyclohexylcarbodiimide solution, on the other hand, is metered by the metering pump and the concentration of the reagent must be calculated for each run. Since the holdup volume and the total volume pumped are known, the actual volume of diimide solution delivered into the vessel can be calculated. The required quantity of dicyclohexylcarbodiimide is dissolved in this volume of methylene chloride. The total volume of solution prepared at one time depends on the number of amino acids to be added.

In a typical diimide cycle, the instrument first washes the resin three times with acetic acid by means of three sets of pumping, shaking, and outlet steps. The metering pump always stops at the end of an exhaust stroke to minimize solvent mixing, and the shaker always stops with the vessel in the upright position to make the following filtering (outlet) step possible. During the third of these outlet steps, the solvent valve advances to posi-

tion 2, and the HCl-acetic acid reagent is then pumped into the vessel. The 30-minute reaction period necessary for complete removal of the tert-butyloxycarbonyl protecting group is obtained by use of three successive 10-minute shaking steps.

After this deprotection step, the resin is washed three times with acetic acid to remove hydrogen chloride, three times with ethanol to remove acetic acid, and three times with dimethylformamide. A 10-minute shaking period with triethylamine in dimethylform- 10 amide serves to neutralize the hydrochloride of the amino acid on the resin, thus liberating the free amine in preparation for coupling with the next protected amino acid. Triethylammonium chloride and excess triethylamine are removed by three washes with dimeth- 15 ylformamide, and prepare the resins for the coupling step. The t.—BOC amino acid solution is then pumped into the vessel in a 30-second pumping step. On the next step (rinse), the pump draws one more stroke of air, then three strokes of methylene chloride to flush 20 the amino acid line.

-The next step is a 10-minute shaking operation to allow the amino acid to soak into the resin beads. During this step, the solvent valve advances to the diimide position. At the next step, diimide solution is pumped 25 for 30 seconds, and then the rinse step adds one more stroke of diimide solution and three strokes of methylene chloride. The coupling reaction then takes place during a 2-hour shaking cycle. After the coupling reaction, by-products and excess reagents are removed by 30 three washes in methylene chloride and two washes in ethanol.

If the end-of-cycle switch is set in the hold position, the instrument stops after the third ethanol wash and the resin is left suspended in ethanol. If this switch is in 35 the "go" position, the drum returns to the beginning of the cycle and proceeds to carry out the next cycle of operation. The apparatus will continue to operate for approximately 24 hours until the coupling cycle of the sixth amino acid has been completed. Then the end-of- 40 run microswitch stops the apparatus. To continue the run, the amino acid reservoirs are washed (solvents are added to the reservoirs and drawn through the amino acid valve and the solvent valve to the waste flask through the three-way stepcock). The amino acid res- 45 ervoirs are then refilled with the proper new solutions, the reagent and solvent reservoirs are replenished if necessary. The amino acid valve is set by a switch to position 12. The drum is then stepped manually back 50 to step 1 to start the coupling of the next six amino acid residues.

Using this apparatus, an active ester coupling cycle may be accomplished instead of a diimide cycle using

some different solvents and reagents with a change in order and setting the timers.

When the synthesis of the desired amino acid sequence has been completed, the peptide-resin is removed from the reaction vessel with the aid of ethanol, filtered, and dried. Weight gain of the resin during the synthesis provides an indication of the amount of peptide incorporated. The peptide is cleaved from the resin with HBr-trifluoroacetic acid and subjected to a suitable purification procedure.

The peptide of the present invention was synthesized by the solid phase method as outlined above and described by Merrifield et al. The peptide was synthesized on the Beckman Model 990 Peptide Synthesizer by the Beckman Company (Palo Alto, Calif.). The resin used was 1% cross-linked chloromethylated divinylbenzene polystyrene beads. Coupling of the resin was performed using t-butyloxycarbonyl amino acid in the presence of dicyclohexycarbodiimide in methylene chloride. The amino acid-resin was deblocked with trifluoroacetic acid in methylene chloride, and neutralized with triethylamine. Following the addition of the thirty fourth residue to the chain, the peptide was removed from the resin with liquid hydrogen fluoride.

In the drawings, and throughout this application, standard abbreviations are used according to the nomenclature:

Serine	Ser
Valine	Val
Glutamic acid	Glu
Isoleucine	Ile
Glutamine	Gln
Leucine	Leu
Methionine	Met
Histidine	His
Glycine	Gly
Asparagine	Asn
Lysine	Lys
Arginine	Arg
Trytophan	Trp
Phenylalanine	Phe

Other abbreviations are:

Boc	t-butyloxycarbonyl
Bpoc	2-(p-biphenylyl)-isopropyloxycarbonyl-
But	t-butyl
DCCl	dicyclohexylcarbodiimide
Hobt	1-hydroybenzotriazole
Trt	Trityl-
Z	benzyloxycarbonyl-

THE CLASSICAL SYNTHESIS

The peptide was also synthesized by a team at Ciba-Geyz AG (Bosle, Switzerland) using the classical method described by Bodansky et al. Intermediate products III, IV, VI, VIII, X and XII were prepared by standard methods. All the intermediate products are designated by the numbers III–XII in Table I.

TABLE I

FORMULAS OF THE PROTECTED INTERMEDIATE PRODUCTS III–XII

No. Sequence   Formula

III 29–34 H-Gln-Leu-Val-His-Asn-phe-OBut

            H⊕ Boc Boc Boc
            | | | |
IV 25–28 Z-Arg-Lys-Lys-Lys-O⊖

            H⊕ Boc Boc Boc   H⊕
            | | | | |
V 25–34 H₂Arg-Lys-Lys-Lys-Gln-Leu-Val-His-Asn-phe-OBut.3Cl⊖

            OBut H⊕
            | |
VI 18–24 Bpoc-Met-Glu — Arg-Val-Gln-Trp-Leu-O⊖

TABLE I—Continued

FORMULAS OF THE PROTECTED INTERMEDIATE PRODUCTS III-XII

No. Sequence   Formula

```
                OBut H⊕                   H⊕ Boc Boc Boc            H⊕
                 |                          |   |   |                |
                 ⊕|                         |   |   |                |
VII 18-34  H₂-Met-Glu—Arg-Val-Gln-Trp-Leu-Arg-Lys-Lys-Lys-Gln-Leu-Val-His-Asn-phe-OBut-4Cl ⊖
```

```
             Boc          But
              |            |
VIII 13-17  Bpoc-Lys-His-Leu-Asn-Ser-NHNH₂
```

```
             Boc H⊕       But     OBut H⊕              H⊕ Boc Boc Boc
              |  |         |       |   |                |   |   |   |
IX 13-34  H₂-Lys-His-Leu-Asn-Ser-Met-Glu—Arg-Val-Gln-Trp-Leu-Arg-Lys-Lys-Lys-Gln-
              |
              H⊕
              |
          Leu-Val-His-Asn-phe-OBut.5Cl ⊖
```

```
             OBut
              |
X 4-12  Trt-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-OH
```

```
            OBut              H⊕           Boc H⊕      But     OBut H⊕
             |                 |            |  |        |       |   |
             ⊕|                |            |  |        |       |   |
XI 4-34  H₂-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu—Arg-
             H⊕ Boc Boc Boc
              |  |   |   |
          Val-Gln-Trp-Leu-Arg-Lys-Lys-Lys-Gln-Leu-Val-His-Asn-phe-OBut.6Cl⊖
```

```
            But    But
             |      |
XII 1-3  Boc-Ser-Val-Ser-NHNH₂
```

From the intermediate products III–XII, the final sequence 1-34 (II) was formed. II is the protected sequence 1-34, with the Boc and But protecting groups on position 1, But on positions 3, and 17, OBut on positions 4, 19, and 34, and Boc on positions 13, 26, 27, and 28. II was formed by the scheme:

```
III }
IV  }——→ V  }——→ VII }
         VI }    VIII }——→IX }
                         X }——→XI }
                              XII}——→ II
```

In the following description Rf refers to thin layer chromatography. S denotes silica gel and C denotes cellulose plates.

Sequence 15-34 (V)

Fragments III and IV were combined by means of DCCI—HOBt7. The crude product was precipitated from acetonitrile-water and was chromatographed by thin layer chromatography using silica gel and an acetic ester, pyridine acetic acid, water (61:21:6:11) solvent system (system 100), Rf (S=0.32). The Z-group was removed by means of catalytic hydrogenation over Pd/C. 3 eq. of HCl were simultaneously added. V was obtained as the trihydrochloride.

(7). Konig. W. & Geiger. R., Chem. Ber., 103, 788 (1970).

Sequence 18-34 (VII).

Coupling of V with the fragment 18-24 (VI) by means of DCCI-HOBt produced the Bpoc-derivative of VII. This was then purified by means of counter-current-distribution in a methanol/0.1 M aqueous ammonium acetate (pH-7.0)/chloroform/carbon tetrachloride 10:4:7:3 system (K=0.33), Rf(S)=0.16 in system 100. Separating the Bpoc-group by means of HCl in trifluroethanol produced the tetrahydrochloride VII.

Sequence 13-34 (IX).

VII was condensed with the azide, produced from VIII and then purified. the Bpoc-derivative thus obtained from IX by means of counter-current-distribution in the methanol/ammonium-acetate/chloroform/carbon tetrachloride system already described (K=0.65), Rf(S)=0.40 in a 2-butanol/acetic acid/water (67:10:23) system (system 96),=0.30 in system 100. the Bpoc-group was again removed with HCl in trifluoroethanol and IX as the pentahydrochloride, Rf(S)—0.23 (system 96) was obtained.

(8). Honzl, J. & Rudinger, J., Coll. Czechoslov. Chem. Commun. 26, 2333 (1961).

Sequence 4-34 (XI).

IX was then coupled with X by means of DCCI-HOBt and the trityl-derivative from XI was the purified by means of counter-current-distribution (system as in IX), K-0.35; Rf(S)=0.28 in system 100. The trityl group was then removed by HCl in trifluoroethanol to the hexahydrochloride of XI, Rf(S)=0.36 in system 96.

Protected Sequence 1-34 (II).

XI was coupled, according to Honzl and Rudinger with the azide which was produced from XII, and the raw product in a methanol/.2M aqueous ammonium acetate (pH=4.75)/chloroform/carbon tetrachloride 10:3:8:4 system (K-0.21); Rf(S)=0.43 in system 96; =0.30 in system 100.

I, Free Human PTH.

The protecting groups were removed from II by means of concentrated hydrochloric acid (10 min., at 0°C) and the hydrochloride of the peptide (I) was added to the acetate via ion-exchange. The peptide thus obtained contained only very small amounts of by-products, mainly a mixture of a methionine-S-oxide-derivative.

Characterization:

Rf(C)=0.36 in a 1-butanol/pyridine/acetic acid/water, 38:20:5:24, system (system 151); =0.54 (system 54). Thin layer electrophoresis in HCl in trifluoroethanol, pH-1.9, 90 min., 16 V/cm, running path of 6cm to the cathode. Distribution Coefficient K=0.12 (n-butanol/0.2M aqueous ammonium acetate (pH=4.75)/methanol 4:4:1).

Amino-Acid-Analysis

(Hydrolysis 15 hours, 118°, 6N HCl) Trp 0.51 (1) (the content of a Trp residue in unhydrolyzed I resulted in a uv spectrum λmax=280,288nm); Lys 3.85 (4); His 2.75 (3); Arg 1.88 (2); Asp 3.05 (3); Ser 2.47 (3); Glu 5.06 (5); Gly 1.07 (1); Val 3.16 (3); Met 1.96 (2); Ile 1.03 (1); Leu 4.75 (5); Phe (base value) 1.00.

Methionine-S-Oxide-Derivative:

a. mixture of the Met^8- and Met^{16}-mono-S-oxide (I in 0.6% aqueous H_2O_2, 3 min. 25°) Rf(C)=0.29 in system 151; =0.45 in a 1-butanol/pyridine/acetic acid/water, 38:24:8:30, system (system 101); =0.48 in a 2-butanol/2-propanol/9% chloroacetic acid, 58:8:34:(v/v), system (system 54).

b. $Met^{8,16}$-di-S-oxide (I in 0.6% aqueous H_2O_2 45 min., 25°); Rf(C)=0.21 in system 151; =0.39 in system 100; =0.43 in system 54.

Biological Activity.

I showed in the thyro-parathyroid ectomy rat, 2 hours after intraveneous injection, in dosages of 100 and 500 μg, a clear increase of the calcium concentration in the serum.

What is claimed is:

1. The peptide represented by the L-isomers of:
H₂N-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Gln-Trp-Leu-Arg-Lys-Lys-Lys-Gln-Leu-Val-His-Asn-Phe-R, wherein R is a carboxyl group.

* * * * *

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SOLUTION SYNTHESIS OF [ASN^{76}]-HUMAN PARATHYROID HORMONE(1-84)

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Human parathyroid hormone, hPTH(1-84), was synthesized by the conventional solution procedure applying the maximal-protection approach.   All protecting groups were removed simultaneously by the HF method.   The product was purified by CM-cellulose column chromatography, gel-filtration on Sephadex G-50 and in the final stage, by reversed phase HPLC. The structure of the final product was confirmed not only by HPLC analysis but also by peptide mapping of tryptic digests on HPLC.   The present product showed 350(249-480) IU/mg on in vitro rat renal adenylate cyclase assay.

The amino acid sequence of human parathyroid hormone, hPTH, was originally determined by Keutmann et al.(1) in 1978 as a single-chain polypeptide with 84 amino acid residues, and the total synthesis was successfully done for the first time by our research group in 1981 (2).   Immediately after our synthesis, the structure was revised by Hendy et al. (3) to have Asn instead of Asp at position 76 in Keutmann's structure, based on deduction from sequence analysis of cDNA cloned for human preproPTH.   This communication reports the solution synthesis of [Asn^{76}]-hPTH(1-84) and the characterization of the product.

MATERIALS AND METHODS

Materials.   DPCC-treated trypsin(EC 3.4.21.4.) was purchased from Sigma Chemicals Co., and amino peptidase-M(EC 3.4.11.2.) was purchased from Pierce Chemical Co.   Boc-amino acids and other reagents for peptide synthesis were obtained from Peptide Institute, Inc., Osaka, Japan.

Abbreviations: WSCI, water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBT, 1-hydroxybenzotriazole; Pac, phenacyl ester; DMF, N,N-dimethylformamide; DPCC, diphenyl bamyl chloride.

Peptide Synthesis.   [Asn[76]]-hPTH was synthesized by the conventional solution procedure as shown in Fig. 1.   Condensation reaction of each segment was carried out in DMF or N-methylpyr-rolidone by the WSCI/HOBT method after removal of the terminal Boc- or Pac group.   The fully protected product was deprotected by the HF method using an HF reaction apparatus, Protein Research Foundation Type I.   The crude product was purified on a column of CM-cellulose, Sephadex G-50 and then by reversed-phase HPLC.

Reversed-Phase HPLC.   HPLC was performed on a Hitachi Liquid Chromatograph Model 638 equipped with a column of Nucleosil $5C_{18}$ (150 x 4 mm).   All runs were performed at ambient tempera-ture at a flow rate of 1.0 ml/min.   Other conditions are given in each figure legend.

HPLC Mapping of Tryptic Digests.   A solution of Peptide (40 µg) in 40 µl of water were treated with a water-solution of DPCC treated trypsin(2.5 µg/5 µl) at 37°C; pH of the mixture was 6. After 30 min; 12 µl of the whole mixture was applied to the reversed-phase HPLC system.

Biological activity.   Biological potency of hPTH was measured by in vitro assay of the rat-kidney adenylate cyclase activity following the procedure developed by Marcus and Aurbach (4) using WHO bovine PTH(1-84) as the standard.

RESULTS AND DISCUSSION

The principle of the present synthesis is based on the maximum protection strategy using stable protective groups at the side chains (5).   The whole molecule was assembled with 13 segments by the route shown in Fig. 1.   Boc-amino acids developed for Merrifield's solid phase procedure were used for stepwise synthesis of each segment in solution, which was started from the C-terminal amino acid Pac ester by the WSCI/HOBT method or by the active ester method.   Each segment was also coupled by the WSCI/HOBT method after removal of the terminal Boc or Pac group; the latter group was removed by warming the peptide with Zn powder in acetic acid.   No particular difficulty was encountered in the segment condensation reactions in solution when DMF or N-methyl-pyrrolidone was used as the solvent.

The fully protected 84-peptide thus obtained was treated with HF at 0°C for 1 hr in the presence of anisole, methionine, dimethylsulfide and ethanedithiol as scavengers.   The crude
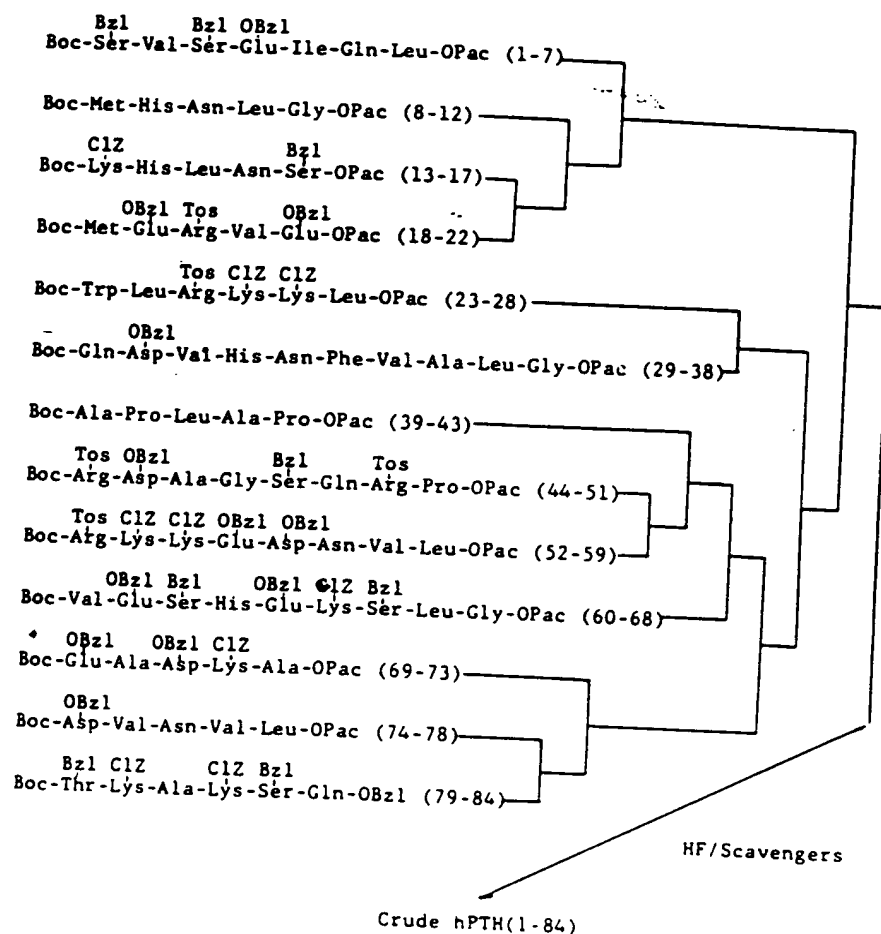
Fig. 1. Coupling route for the synthesis of [Asn'⁶]-hPTH(1-84).

product was fractionated on a CM-cellulose column using ammonium acetate solution with linear gradient concentrations from 0.05 M at pH 5 to 0.4 M at pH 6, and then by gel-filtration on Sephadex G-50 using 1 M acetic acid as the solvent. The main peak of the Sephadex chromatogram was developed on a gradient HPLC system (Fig. 2); the main peak here was collected from several different runs and rechromatographed on an isocratic HPLC system; the elution profile is shown in Fig. 3. The homogeneity of each ...ion of the broad peak was checked with a similar HPLC system under analytical conditions; fractions showing a single peak on
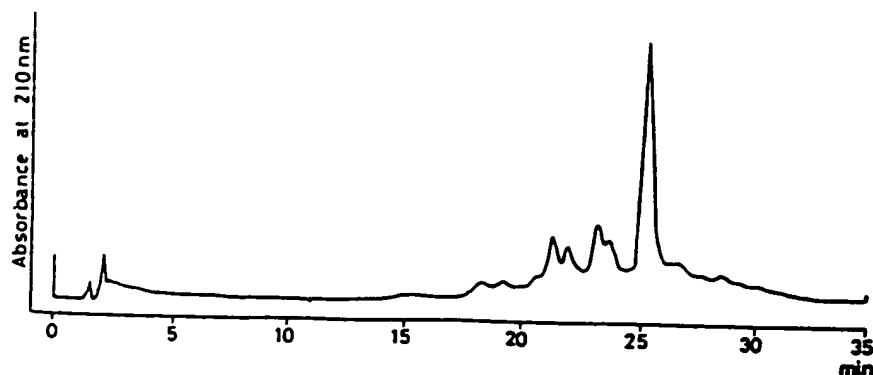
Fig. 2. HPLC profile of crude product obtained after gel-filtra-
tion on Sephadex G-50.
Eluant: 0.1 M NaCl(pH 2.4) containing MeCN, which was
gradiently increased from 27.5% to 40%.

analytical HPLC were collected and lyophilized to obtain a final
product.

Amino acid analysis of an acid hydrolyzate and an Ap-M digest
revealed that the product contained all component amino acids in
the expected ratios (Table 1).   When the final product was
treated with a dilute aqueous hydrogen peroxide solution, the
reaction mixture showed four peaks on HPLC including that of the
intact molecule (Fig. 4).   The formation of three additional
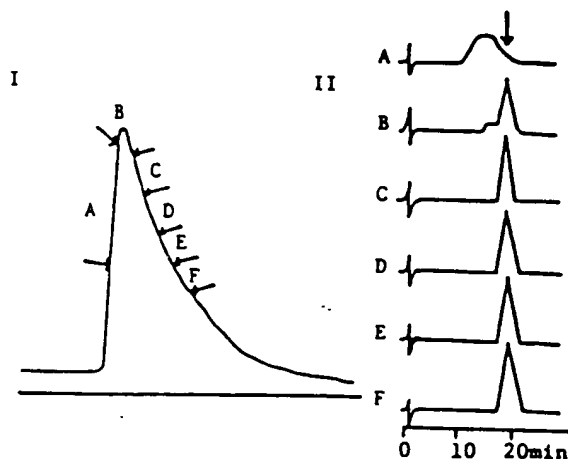
Fig. 3. Separation of final product, h-PTH(1-84), on HPLC.
I. Isocratic HPLC profile of collected materials.
II. Purity check of each fraction in I under analytical
conditions; fractions C,D,E and F were pooled for
lyophilization as the purified material. Eluant: 31%
MeCN in 0.1 M NaCl(pH 2.4).

Table 1. Amino acid analyses of synthetic [Asn^{76}]-hPTH.

Amino Acid	Expected	6 N-HCl	Ap-M
Lys	9	9.36	8.92
His	4	3.60	3.60
NH$_3$	9	10.08	
Arg	5	5.15	
Asp	5		3.76
Asp+Asn	10	10.00	
Thr	1	0.99	
Thr+Gln	5		3.85
Ser	7	5.74	
Ser+Asn	12		11.40
Glu+Gln	11	10.32	
Cit+Glu	12		10.80
Pro	3	2.74	3.24
Gly	4	4.00	4.00
Ala	7	7.07	6.86
Val	8	7.76	7.52
Met	2	1.20	1.12
Ile	1	0.77	0.90
Leu	10	9.90	9.20
Phe	1	1.01	1.04
Trp	1	0.53	0.52

peaks may be explained by the partial or complete oxidation of
the Met residues to the sulfoxides since the hPTH molecule
contains two Met residues at positions 8 and 18.   This observa-
tion proved that the purified material was free of contamination
from oxidized peptides.
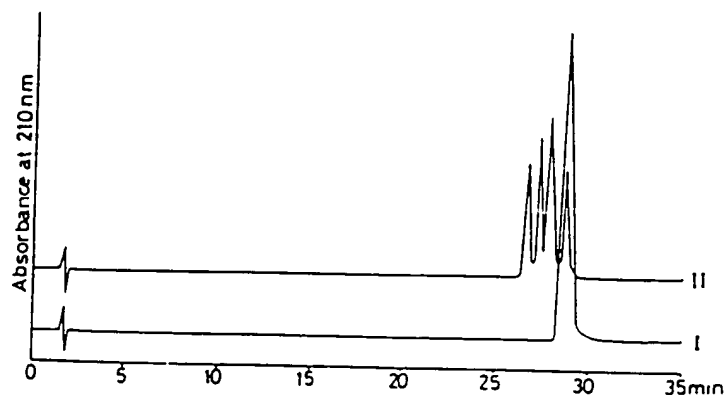
Fig. 4. HPLC profile of purified synthetic hPTH(1-84) and its
hydrogen peroxide-treated products.
I. hPTH(1-84) II. Oxidized products. Eluant: 0.1 M NaCl
(pH 2.4) containing MeCN, which was gradiently increased
from 10% to 50%.
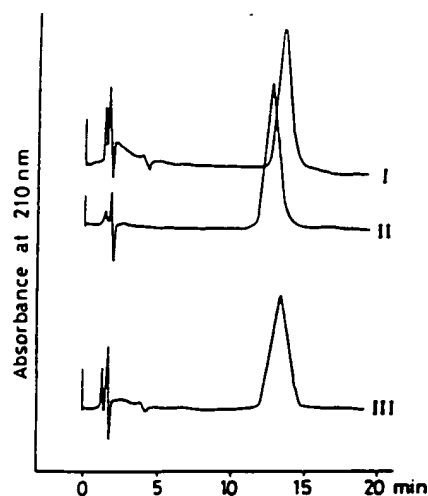
Fig. 5. Separation of Asn- and Asp-hPTH(1-84) on HPLC.
I. [Asp^{76}]-hPTH   II. [Asn^{76}]-hPTH   III. Mixture of Asp-
and Asn-PTH.   Eluant: 31% MeCN in 0.1 M NaCl (pH 2.0).

Our HPLC system showed enough resolution power for analyzing analogs of hPTH(23-84) containing Asp or Asn at position 76. However, the system failed to separate [Asn^{76}]- and [Asp^{76}]-hPTH(1-84) molecules into two peaks when they were injected onto the column as a mixture (see Fig. 5).   Thus, the homogeneity had to be checked by other means. HPLC mapping of the trypsin digest was compared with those of some other synthetic peptides as references (Fig. 6).   All peaks in the chromatograms were assigned as shown in the figure.   In the chromatogram of the present product, all the expected fragments were observed and no unwanted peaks appeared.   The present product might have been contaminated by D-Glu^{22}-containing peptide since the last coupling reaction was performed between segments(1-22) and (23-84), but no D-Glu^{22}-containing fragment(21-25) was detected by tryptic mapping within the range of resolution.   Thus, We concluded that our present product was reasonably homogeneous.   The product showed 350(249-480) IU/mg in the rat-kidney adenylate cyclase assay.   The specific optical rotation value was observed to be: $[\alpha]_D^{20}$ -89.2°(c 0.2, 1% AcOH).

498
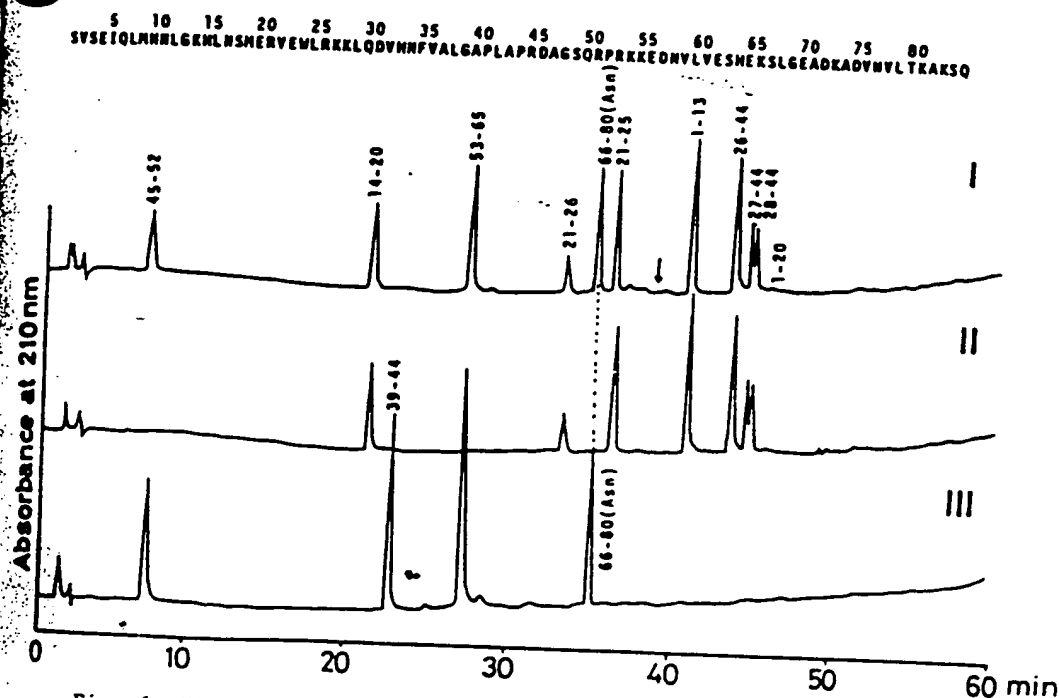
Fig. 6. HPLC mapping of a tryptic digest of [Asn⁷⁶]-hPTH in comparison with those of some other synthetic peptides. I. Asn-hPTH(1-84). II. hPTH(1-44)   III. Asn-hPTH(39-84) Arrow indicates the position of [D-Glu²²]-containing fragment(21-25). Eluant: 10 mM phosphate buffer(pH 2.6) containing 50 mM Na₂SO₄ and MeCN. which was increased gradiently from 2% to 50%.

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CHROMSYMP. 538

COMPARISON OF REVERSED-PHASE AND CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SEPARATING CLOSELY RELATED PEPTIDES: SEPARATION OF ASP[76]-HUMAN PARATHYROID HORMONE (1-84) FROM ASN[76]-HUMAN PARATHYROID HORMONE (1-84)

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SUMMARY

Cation-exchange high-performance liquid chromatography (CE-HPLC) was compared with ordinary reversed-phase high-performance liquid chromatography (RP-HPLC) for separating closely related peptides. Some synthetic samples of bradykinin and angiotensins, which were homogeneous according to RP-HPLC, were found to be inhomogeneous when analyzed by CE-HPLC. On the other hand, diastereomeric peptides could be separated much more efficiently by RP-HPLC than by CE-HPLC. These results indicated that the purity of synthetic peptides chould be checked not only by RP-HPLC but also by ion-exchange HPLC. In the case of human parathyroid hormone (hPTH), baseline separation of Asp[76]-hPTH from Asn[76]-hPTH by RP-HPLC was not possible, but was by CE-HPLC. Using this method we confirmed that the Asn residue in hPTH at position 76 could not be converted into the Asp residue under the conditions used to isolate and purify it from human organs.

INTRODUCTION

In 1978, Keutmann *et al.*[1] found the amino acid sequence of human parathyroid hormone (hPTH) to be a linear peptide with 84 amino acid residues when they applied Edman degradation reactions to an isolated hormone. The structure of the same peptide was deduced by Hendy *et al.*[2] from sequence analysis of the cDNA; both structures were identical except for the residue at position 76, which was thought to be Asp from the Edman degradation analysis and Asn from the DNA analysis. When the new structure was reported, the previous one with Asp at position 76 was suspected to be an artifact which might have been formed by spontaneous deamidation of the Asn residue during isolation or purification of the natural peptide. To verify this, we synthesized both Asn[76]-hPTH and Asp[76]-hPTH by the solution procedure[3,4], and examined their separation by ordinary reversed-phase high-performance liquid chromatography (RP-HPLC). Under isocratic conditions, the hormones

were eluted very close together but with clearly different retention times. However, when injected into the same column as a mixture, the peptides were eluted together as a rather broad peak[4,5]. In the present study we have tried to establish conditions for separating such closely related peptides by HPLC.

Recently, ion-exchange type columns have been introduced for HPLC and their usefulness in separating peptides and proteins has been reported[6,7]. In order to elucidate the characteristic features of the new technique, we compared the resolving power of cation-exchange HPLC (CE-HPLC) with that of ordinary RP-HPLC by using some synthetic peptides as test samples. We also used the CE-HPLC technique to find the conditions for separation of the two hPTH analogues, and then applied them to determine whether or not Asp^{76}-hPTH is an artifact formed during isolation of hPTH from organs.

EXPERIMENTAL

Materials

Commercial samples of angiotensin I (AngI) and bradykinin (BK) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), UCB Bioproducts (Bruxelles, Belgium) and Bachem (Bubendorf, Switzerland). Other peptides were synthesized by solution procedures in our laboratory using previously reported methods[8]. The reagents and solvents for chromatography were of HPLC-reagent grade. The water used was distilled in a Toyo Aquarius Model GS-20N still (Toyo Kagakusangyo, Tokyo, Japan).

CE-HPLC

CE-HPLC was carried out on a Shimadzu liquid chromatograph Model LC-4A (Kyoto, Japan) equipped with a Rheodyne 7125 syringe-loading sample injector, a Shimadzu variable-wavelength UV detector Model SPD-2AS, a Shimadzu column oven Model CTO-2AS and a Shimadzu data processor Chromatopac C-R2AX. The column (250 × 4.6 mm I.D.) was packed with a cation-exchange resin, TSK gel CM-2SW (Toyo Soda, Tokyo, Japan). Two solvent systems were used: A, 10% acetonitrile in 20 mM sodium phosphate buffer (pH 6.0); B, 10% acetonitrile in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. Elution was performed at 42°C at a flow-rate of 0.8 ml/min; other conditions are as stated in the Figs.

RP-HPLC

RP-HPLC was performed on a Hitachi liquid chromatograph Model 638-30 equipped with a multi-wavelength UV monitor Model 635M (Tokyo, Japan); the column (150 × 4.0 mm I.D.) was packed with Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.). Chromatography was carried out at a flow-rate of 1.0 ml/min at ambient temperature unless stated otherwise.

Test of conversion of Asn^{76}-hPTH into Asp^{76}-hPTH

Asn^{76}-hPTH (15 μg) was dissolved in 75 μl each of water, 0.1 M ammonium acetate buffer (pH 2.5, 5.0 or 7.5), 0.1 M ammonium hydrogen carbonate buffer (pH 8.7) or 4% trichloroacetic acid (TCA). After the solution had been kept for 1 day or

5 days at room temperature, 35 μl from each solution were lyophilized twice; the residue was dissolved in 20 mM sodium phosphate buffer (pH 6.0) and subjected to HPLC. With the TCA solution, the whole mixture was lyophilized after it had been kept for 1 day at room temperature, and the residue was subjected to HPLC after it had been dissolved in a 20 mM sodium phosphate buffer (pH 6.0).

RESULTS AND DISCUSSION

As reported previously[9], various angiotensin II (AngII) analogues, such as β-Asp[1]-, D-Asp[1]-, D-Tyr[4]-, Val[5]-, Leu[5]-, des-Ile[5]-, D-His[6]- and D-Phe[8]-AngII, were clearly separated by RP-HPLC, except for β-Asp[1]-AngII, which is always eluted together with Asp[1]-AngII in our RP-HPLC system. The separation could be slightly improved by increasing the pH of the buffer system, but it was far from a baseline separation. CE-HPLC enabled a clear separation of the two AngII analogues as shown in Fig. 1. These results indicate that the purity of synthetic peptides containing Asp residue(s) should be checked not only by RP-HPLC but also by CE-HPLC. Particularly important is the detection of β-Asp-containing peptide since some Asp residue(s) in peptides have a great tendency to be converted into β-Asp residue(s) during various steps of their synthesis. We applied these techniques to test the homogeneity of commercially available samples of AngI. As expected in RP-HPLC, all samples looked homogeneous, but in CE-HPLC they were found to be contaminated by β-Asp-AngI, in the range of 2.3–17%, and by various other minor contaminants; a typical elution pattern is shown in Fig. 2.
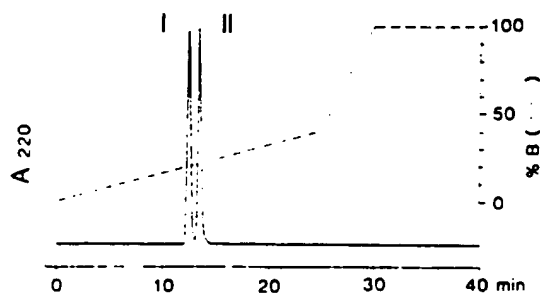
Fig 1. Separation of β-Asp[1]- and Asp[1]-angiotensin II by CE-HPLC. Column: TSK gel CM-2SW (250 × 4.6 mm I.D.). The column was equilibrated with solvent A, and then the peptide was eluted with solvent B using a gradient as shown in the figure. See text for other details. Peaks: I = β-Asp[1]-AngII; II = Asp[1]-AngII

Next, we tested the homogeneity of commercial bradykinin (BK) samples since this peptide is also widely available. Only one commercial sample gave a single peak in RP-HPLC, but the shape was somewhat broader than that of our standard sample. Even the best sample gave a relatively large extra peak in CE-HPLC as shown in Fig. 3; the peak area was about 13% of the total. The structure of the component in the side peak was examined by chymotryptic mapping in HPLC and by amino acid analysis; it was confirmed to contain a normal bradykinin sequence (1–8), identical with that isolated from an authentic sample, together with one Orn residue instead of one Arg residue. Thus, the main component of the side peak was Orn[9]-BK.
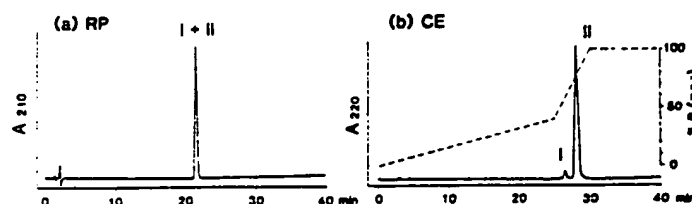
Fig. 2. RP- and CE-HPLC profiles of a commercial AngI. (a) RP-HPLC. Column: Nucleosil 5C18 (150 × 4 mm I.D.). Gradient: 10–60% acetonitrile in 10 mM H_3PO_4–K_2HPO_4 (pH 2.6) containing 50 mM Na_2SO_4. (b) CE-HPLC. Column and elution conditions as in Fig. 1. Peaks: I = β-Asp[1]-AngI; II = Asp[1]-AngI.
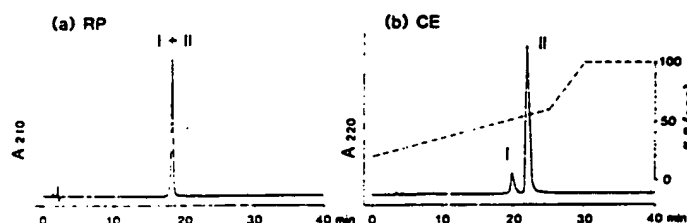
Fig. 3. RP- and CE-HPLC profiles of a commercial bradykinin. (a) RP-HPLC. Column and elution conditions as in Fig. 2. (b) CE-HPLC. Column as described in Fig. 1. It was equilibrated with 20% B in A before applying the peptide; then the peptide was eluted with solvent B using a gradient as shown. Peaks: I = Orn°-BK; II = BK.

Today, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system: the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products.

With regard to the separation of peptide diastereomers, the usefulness of RP-HPLC has been recognized by many groups, and several examples of diastereomer separation have been reported by Dizdaroglu's group[10,11], who used a weak anion-exchange column. We also tried to separate D-Ala[73],Asp[76]-hPTH (39–84) from the L-peptide by our CE-HPLC, but we were unsuccessful despite the good separation of the same compounds in our RP-HPLC system[5]. A similar result was obtained with a pair of shorter peptides. D-Glu[22]-hPTH (18–28) and its L-isomer. However, in the case of human growth hormone-releasing factor (hGRF), D-Leu[22]-hGRF(1–44)-NH$_2$ was clearly separated from its original L-peptide not only by RP-HPLC[12] but also by CE-HPLC as shown in Fig. 4. From these observations, we concluded that some diastereomeric isomers can be separated by IE-HPLC but the system may not be suitable in general for detecting racemization during peptide synthesis.

Finally, we examined the separation of Asp[76]-hPTH (1–84) from Asn[76]-hPTH by the CE-HPLC system. Clear separation was achieved even when a 1:1 mixture was applied, as shown in Fig. 5. This CE-HPLC technique was then used to detect possible conversion of the Asn residue into Asp under the various conditions used to isolate and purify natural hPTH; that is, the peptides were kept in water, 0.1 M ammonium acetate solution (pH 2.5, 5.0 or 7.5), 0.1 M ammonium hydrogen carbonate solution (pH 8.7) or 4% TCA solution for a maximum of 5 days at room

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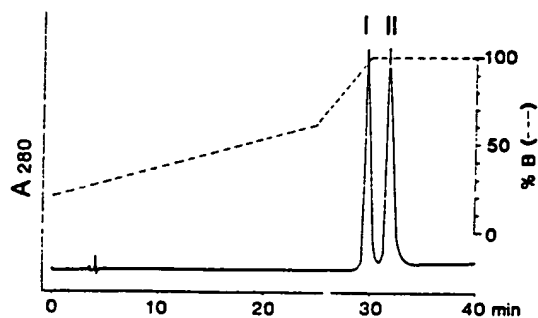
Fig. 4. Separation of D-Leu²²-hGRF(1–44)-NH₂ and the all-L-peptide by CE-HPLC. Column and elution conditions as in Fig. 3b. Peaks: I = D-Leu²²-hGRF(1–44)-NH₂; II = hGRF(1–44)-NH₂.

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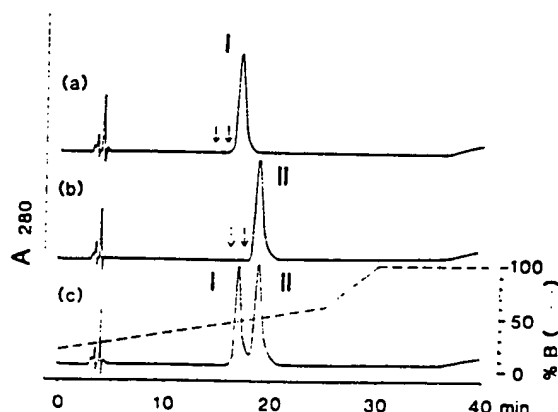
Fig. 5. Separation of Asp⁻⁶- and Asn⁻⁶-hPTH(1–84) by CE-HPLC. (a) Application of compound I only; (b) application of compound II only; (c) application of a mixture of I and II. Column and elution conditions as in Fig. 3b. Peaks: I = Asp⁻⁶-hPTH(1–84); II = Asn⁻⁶-hPTH(1–84). Arrows indicate the peak positions of Met(O)-containing peptides

temperature¹³. No clear evidence of conversion of the Asn residue into Asp was obtained. Recently, Gleed *et al.*¹⁴ confirmed. by using radioimmunoassay, that our synthetic Asn⁻⁶-hPTH had better cross-reactivity with an antibody, raised against an isolated natural hPTH. than our Asp⁻⁶-hPTH. Furthermore. the cross-reaction curve of Asn⁻⁶-hPTH was completely parallel to that of the natural hormone. From this evidence. together with the results of the above conversion tests, we concluded that the Asp⁻⁶ structure claimed for hPTH might have been due to misreading the results of the original Edman degradation reactions.

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Human Parathyroid Hormone: Amino-Acid Sequence of the Amino-Terminal Residues 1-34

(automated Edman degradation/mass spectrometry/calcium metabolism/metabolic bone disease)

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ABSTRACT Human parathyroid hormone has been isolated in highly purified form from human parathyroid adenomas. The primary sequence of the amino-terminal residues of the human hormone was obtained by automated degradation with a Beckman Sequencer. The phenylthiohydantoin amino acids were identified by gas chromatography and mass spectrometry. The first 34 residues of human parathyroid hormone differ from the bovine hormone by six residues, and from the porcine hormone by five residues. The amino-terminal residue is serine, similar to the porcine parathyroid hormone; bovine parathyroid hormone contains an amino-terminal alanine. Human parathyroid hormone contains two methionine residues, similar to the bovine species, whereas porcine parathyroid hormone contains a single methionine residue. Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 16, a glutamine at position 22, lysine at position 28, and a leucine at position 30.

During the last few years, a significant core of information has been obtained by several laboratories on the chemistry, biosynthesis, and secretion of parathyroid hormone (PTH). These studies have indicated that this hormone is initially synthesized as a prohormone, proparathyroid hormone (1-4). Proparathyroid hormone contains about 106 amino acids, and has an apparent molecular weight of 12,500 (4). The prohormone is rapidly converted into the storage or glandular form of the hormone, which consists of 84 amino acids and has a molecular weight of 9500. The complete amino-acid sequences of the 84 amino-acid parathyroid hormone from bovine (5, 6) and porcine (7) species have been reported. After appropriate physiological stimuli, the 9500 molecular weight form of the parathyroid hormone is secreted into the circulation (8). Shortly after entering the peripheral circulation, the glandular form of the hormone is cleaved into smaller fragments. Gel filtration of human hyperparathyroid serum by several investigators has revealed a major immunoreactive fragment(s), with a molecular weight of 5000-8000, and several minor components (8-10). Immunochemical heterogeneity of the circulating human parathyroid hormone, presumably due to the different molecular forms of PTH, was initially reported by Berson and Yalow (11), and has been confirmed by others (12, 13). The specific site(s) of cleavage in the 84-amino-acid polypeptide chain of the parathyroid hormone in the general circulation is unknown. A biologi-

cally active peptide fragment of bovine PTH, prepared by dilute acid cleavage, has been reported (14, 15), a result indicating that the intact 84-amino-acid polypeptide is not needed for biological activity. This peptide has been identified as the amino-terminal peptide of the hormone, and is composed of the initial 30 residues of the sequence (15). Synthetic peptides of the first 34 residues of the bovine hormone (16) and the initial 30 residues of the porcine hormone (17) have been prepared and are biologically active, thereby confirming the localization of the biologically active region of the parathyroid hormone to the amino-terminal third of the 84-amino-acid polypeptide chain.

The purpose of this communication is to report the amino-terminal sequence of the first 34 residues of human parathyroid hormone, and to compare the amino-terminal sequence of the human hormone to that of the bovine and porcine species.

MATERIALS AND METHODS

The human parathyroid hormone used in these studies was isolated from parathyroid adenomas obtained from patients undergoing surgery for hyperparathyroidism. Dried, defatted parathyroid tissue was initially extracted with 8 M urea in 0.2 N hydrochloric acid, and fractionated with ether, acetic acid, sodium chloride, and trichloroacetic acid (TCA powder) (18). The TCA powder was further purified by gel filtration, followed by ion-exchange chromatography on CM-sephadex with an ammonium acetate gradient. Isolation of the hormone was monitored by radioimmunoassay and disc-gel electrophoresis. The procedures used in the isolation and characterization of the hormone will be described in detail in a separate report.

Amino-acid analyses were performed on a Beckman-Spinco automatic amino-acid analyzer, model 120B or 121 adapted for high sensitivity or with a Durrum model 500 analyzer. Analytical disc-gel electrophoresis was performed in 8 M urea at pH 4.4 (19). Immunoassays were performed by the procedure of Arnaud et al. (20).

Automated Edman degradations were performed with the Beckman Sequencer, model 890B, in 1 M Quadrol buffer. The phenylthiohydantoin (PTH) amino acids were identified by regeneration to the constituent amino acid by hydrolysis with hydroiodic acid for 20 hr at 130° (21), gas-liquid

Abbreviation: PTH, parathyroid hormone.

fluorobutyric acid at each degradation. The results of the degradation of the first 34 residues of the human parathyroid hormone are shown in Fig. 2. The chemical ionization mass spectra of the phenylthiohydantoin (PTH) amino acids obtained at each of the 34 steps in the sequence are shown in Fig. 3. A "quasimolecular" (QM$^+$) or major fragmentary ion is observed in each spectrum (25). At step 12 in the sequence, a quasimolecular ion for glycine (m/e 192) and leucine (m/e 249) are observed (Fig. 3). Quantitation by the gas chromatography method of glycine (0.28 μM) and leucine (0.09 μM) permits definitive identification of glycine as the twelfth amino acid in the sequence, with the leucine resulting from overlap from step 11 (Fig. 2). The ion at m/e 292 and 293 in the mass spectra of step 20 are contaminant ions often observed in variable amounts in the aqueous layer of the Edman reaction. Leucine/isoleucine and lysine/glutamine yield identical masses of m/e 249 and m/e 264, respectively, on chemical ionization mass spectrometry. Lysine, however, can be distinguished from glutamine by the fragmentary ion at m/e 306, as illustrated in the spectra of residues 26, 27, and 28. Lysine/glutamine and leucine/isoleucine were also readily differentiated by gas chromatography on the CFC blend (23) and by electron impact mass spectrometry (24, 25).

These combined results provided a single unique sequence for the first 34 residues of human parathyroid hormone (Fig. 2).

DISCUSSION

The amino-acid sequence of the first 34 residues of human parathyroid hormone is of major importance, since previous studies of the bovine and porcine species have indicated that this is the biologically active region of the native hormone. The first 34 residues of human PTH differ from bovine PTH by six residues, and porcine PTH by five residues (Fig. 4). The amino-terminal 15 residues of human and porcine PTH are identical; however, bovine PTH differs from human and porcine PTH in position 1 and 7, where alanine substitutes for serine and leucine replaces phenylalanine (Fig. 4). In the remaining 16–34 region, human PTH differs from porcine PTH by five residues, and from bovine PTH by four residues (Fig. 4). Human PTH contains two methionine residues—similar to the bovine species—whereas porcine PTH contains a single methionine at position 8 (Fig. 4). The human sequence is unusual in that it contains four consecutive basic residues (arginine residue 25, and lysine residues 26–28). Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 16, glutamine at position 22, lysine at position 28, and a leucine at position 30.

One of the major problems in the clinical assessment of patients with disorders of mineral metabolism has been the difficulties encountered with the radioimmunoassay of human parathyroid hormone. There have been two basic problems with the immunoassay of PTH. The first problem, as discussed above, has been the presence in the peripheral circulation of peptide fragments of the 84 amino-acid polypeptide chain (8–10). Antisera from various laboratories undoubtedly have immunological determinants for different regions of the intact molecule, thus leading to variable and sometimes inconsistent results when applied to the measurement of PTH circulating in human blood (27). In addition, the differentiation by immunoassay of biologically active

FIG. 1. Disc-gel electrophoresis of purified human (left) and bovine (right) parathyroid hormones.

chromatography (22, 23), and mass spectrometry (24–26). Chemical ionization mass spectrometry was performed on a Finnigin mass spectrometer equipped with a PDP-8/e Digital computer, and a Complot Plotter. Isobutane was used as the carrier gas, and the source was maintained at 200°. The samples were applied by a direct insertion probe, and the probe was heated from 30° to 250° over a 90-sec period. Electron impact mass spectrometry was performed on an LKB mass spectrometer, model 9000, with a direct insertion probe and an electron energy of 70 eV.

RESULTS

The purified human parathyroid hormone migrated as a single component on disc-gel electrophoresis, with a mobility identical to that of the bovine parathyroid hormone (Fig. 1). Amino-terminal analysis of the purified peptide by the Edman technique revealed serine.

350 nanomoles of the purified hormone were degraded on the Beckman Sequencer by use of a single cleavage of hepto-
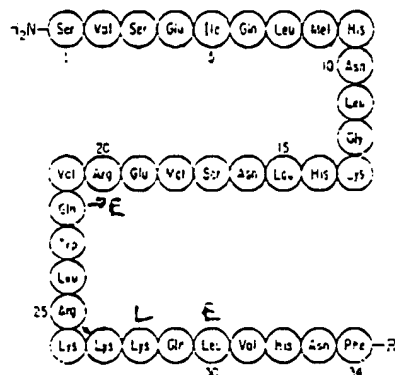

FIG. 2. Amino-acid sequence of the amino-terminal 34 residues of human parathyroid hormone.

FIG. 3. Mass spectra of the phenylthiohydantoin (PTH) amino acids obtained during the automated Edman degradation of the amino-terminal 34 residues of the human parathyroid hormone.

amino-terminal fragments from inactive fragments has so far been impossible. The second difficulty has been the utilization of heterologous assays that use radioactively labeled bovine hormone as the tracer, and antibodies prepared against the bovine or porcine hormone (28–30, 20). The sensitivities of these assays are variable, and depend on the cross reactivity of the particular antiserum with the human hormone. As noted above, the human sequence in only the initial third of the molecule differs from the bovine by six residues and the porcine by five amino acids.

Habener et al. (31) have attempted to circumvent some of these problems with the immunoassay by the development of amino- and carboxyl-specific antisera. These investigators have used an antibody prepared against the bovine hormone, and have absorbed their antiserum with either the synthetic 1–34 bovine fragment, or a 53–84 fragment prepared by chemical cleavage of the native bovine hormone. The amino-terminal specific antiserum was further characterized by displacement with synthetic bovine fragments, and the recognition site of this absorbed antiserum was shown to be directed toward residues 14–19 in the bovine sequence. Using this approach, they have concluded that the major fragment in the human circulation is carboxyl-terminal, and biologically inactive. They were, however, unable to identify the amino-

FIG. 4. Comparison of the amino-acid sequence of the amino-terminal 34 residues of human-, bovine-, and porcine-parathyroid hor-

terminal fragment in the circulation of human subjects. This may be due either to rapid clearance of the amino-terminal fragment from the circulation, or to poor cross-reactivity of the amino-terminal specific bovine antiserum with the amino-terminal region of the human hormone. It is of interest that the human sequence differs in the 14–19 region from the bovine hormone by the substitution at residue 16 of an asparagine for a serine residue (Fig. 4). The significance of this substitution in the human hormone to the results obtained by Habener *et al.* with their amino-terminal specific bovine antiserum is unknown. Canterbury and Reiss have reported results on the nature of the circulating fragment of the parathyroid hormone that are in contrast to those reported by Habener *et al.* Using an antiserum prepared against bovine parathyroid hormone, these investigators have identified three different immunochemical forms of the parathyroid hormone in the peripheral circulation of hyperparathyroid patients (32). The molecular weights of these three components, as determined by gel filtration, were 9500 (presumably glandular PTH), 7000–7500, and 4500–5000. Recently, these investigators have directly assessed the biological activity of these three fragments in a renal adenylate cyclase system (33). Both the 9500 and the 4500–5000 fragment stimulated the adenylate cyclase system, whereas the 7000–7500 component was inactive. These results are consistent with the presence of an amino-terminal biologically active fragment of PTH of about one-half the size of the glandular hormone in human hyperparathyroid serum.

The determination of the amino-terminal sequence of the human parathyroid hormone will now permit the synthesis of peptides based on the human sequence for both clinical and investigative use. Synthetic fragments, as well as chemical analogues, will permit more definitive studies to be performed on the chemistry of the human hormone, including the specific residues and the minimum length of the polypeptide chain that is required for biological activity. In addition, these synthetic fragments will enable investigators to characterize the heterologous antisera currently in use in the immunoassay, and to develop specific antisera directed toward the amino-terminal region of the human hormone. Antisera based on the human sequence will enable more detailed studies to be performed on the nature of the circulating hormone in man, and its role in calcium homeostasis and metabolic bone disease.

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Synthesis of mature human parathyroid hormone in Escherichia coli

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AS 2

Summary

Mature human parathyroid hormone (1-84 AA) was expressed in Escheri-
chia coli. The spacing between ribosome binding sites (RBS) and ATG,
and the use of strong promoters were varied. PTH yield under the con-
trol of the lac-promoter was 140 ug/liter and under the control of the
trp-promoter 200 ug/liter. The PTH had a half-life of 30 minutes in
the log growth phase. The PTH made in E.coli appears identical to
human PTH in a radioimmunoassay and in its biological activity in an
in vitro-assay.

Introduction

Human parathyroid hormone (hPTH) is a protein of 84 amino acids, from
the parathyroid glands. PTH is the principal homeostatic regulator
of blood calcium. At high concentration PTH rapidly increases the bone
decalcification. At a low concentration and with long exposure PTH
stimulates bone formation (1). Human PTH is effective in the treat-
ment of osteoporosis inducing an anabolic response in the bone (2). In
addition PTH may prove effective in the treatment of bone fractures.
We have constructed bacterial plasmids which instruct the synthesis of
biologically active mature PTH in Escherichia coli.

Material and Methods

Bacterial strains and plasmids
We used the E.coli strains 5K-(m+,thr⁻thi,Smr):Glover, SK2124 :
Hautala, SG4121 (F- 1 on 100 gal-blu r-, m+ rec A) : Gottesman,
CSR603 (recA1, uvrA6, phr-1) HB101 (gal-, pro-, leu-, thi-, endoI-,
hsm, hsr, recA, rpsL), JM103 (lac-proΔ), supE, thi, strA, endA,
sbcB15, hsdR4, F'tra D36, pro AB, lacI⁹,ΔZ M15) from P.L. Biochemi-
cals GmbH and the plasmids pBR322 (5) pUR108-1 (U. Rüther, unpub-
lished), pDS1 (6), gift from D. Stüber. pDR540 (7) and pDR720 were
obtained from P.L. Biochemicals GmbH. Unless otherwise indicated
the strains were grown in LB-medium (10g tryptone, 10g yeast extract
and 5g NaCl per liter).

DNA-methodology
Restriction enzymes, T4-ligase and DNA-polymerase (Klenow Fragment)
were obtained from Amersham Buchler, BRL, and Boehringer Mannheim and
used according to the manufacturer's specifications. Treatment with

S1-nuclease (1 unit/μg DNA, 20 min., 20°C) was performed according to
(8). DNA was sequenced according to (9).

Induction of the trp, lac and tac promoters

Strains with the trp-promoter were grown in minimal medium with casa-
mino-acids (M9CA (10)) and induced with 5 μg/ml 3-ß indole acrylic-
acid (IAA). Strains with tac or lac promoters were grown in LB-medium
and induced with 1mM IPTG either continiously or for a 2.5 h period.

Detection of PTH activity in bacterial extracts

1 ml of a 50 ml culture was harvested by centrifugation and resuspen-
ded in 200 μl 3M guanidiniumhydrochloride, 20mM Tris-HCl pH 8, 25mM
glucose, 5mM EDTA and sonicated at 60 watt for 15 sec in ice. Cell
debris was spun down at 8000 g for 1 min. Serial dilutions of the su-
pernatants were analysed by radioimmunoassays by using the PTH kit
from Immuno Nuclear Corporation, and in the adenyl cyclase assay accor-
ding to (11).

Results and Discussion

Isolation of phages containing the human PTH-sequences

We screened a human fetal liver genomic DNA library constructed in the
phage Charon 4A by Lawn et al. (12) probed with a bovine (13) or por-
cine (14) PTH cDNA. Two plaques λ hPTH24 and λ hPTH36 were positive.
Both clones contain the PTH gene coding region and the 3' noncoding
region. The EcoRI-fragment coding for PTH from λ hPTH36 was subcloned
into the EcoRI-site of pBR322 (p20.36) to facilitate DNA-sequence ana-
lysis (Fig. 1).

The first intervening sequence interrupting the 5' noncoding region of
the mRNA is only partially present on the clone we selected. The sec-
ond is 103 bp long and separates the second and third nucleotide en-
coding lysine 29 of Prepro-PTH. The DNA-sequence is identical to the
sequence of the genomic PTH-gene previously described by Vasicek et
al. (4).

Expression of hPTH

For the construction of a functional initiation region we chose a stra-
tegy of sequentially assembling DNA fragments coding for the ribosomal
binding site, the spacer and the initiation codon.
The coding region for hPTH can be isolated as a Sau 3A 980 bp frag-
ment from the subclone p20.36. To restore the first amino acid codon
(Ser) the Sau 3A-sites were filled out with Klenow-Polymerase in the
presence of dGTP and dATP and flush ended with S1-nuclease treatment.
The ATG initiation codon was attached by flush-end ligation of the
synthetic oligonucleotide 5'-CATCGATG-3'. The additional internal

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C) was performed according to

ers

in minimal medium with casa-
5 µg/ml 3-β indole acrylic-
cters were grown in LB-medium
ously or for a 2.5 h period.

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centrifugation and resuspen-
e, 20mM Tris-HCl pH 8, 25mM
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ssays by using the PTH kit
the adenyl cyclase assay accor-

PTH-sequences

DNA library constructed in the
ed with a bovine (13) or por-
and λ hPTH36 were positive.
region and the 3' noncoding
from λ hPTH36 was subcloned
facilitate DNA-sequence ana-

ng the 5' noncoding region of
clone we selected. The sec-
nd and third nucleotide en-
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Fig. 1 shows maps of λ hPTH36 and p20.36. The sequencing strategy is indicated by arrows (open/closed circles mean labelling with T4-Polynucleotide Kinase/Klenow Polymerase).
S=SstI, E=EcoRI, H=HindIII, X=XbaI, ⟶ direction and extent of the sequence determinations.

ClaI-site in the DNA adaptor allows a subcloning into the ClaI-site of pBR322. A tetracycline sensitive clone was selected with the PTH-in-sert oriented counterclockwise from the HindIII-site. A combination of fill-in of dNTPs and S1-treatment of the HindIII linearized plas-mid pEB3 generated DNA fragments with variable distances between the ATG initiation codon and the end of the fragments. Ligation of a sec-ond synthetic DNA adaptor the 5'-TCCCTAGGGA-3' (RBS-linker), carrying a ribosome binding site, onto these fragments creates a series of DNAs with 6-10 bp between the ribosome binding site and the initiation codon ATG. Since the dimeric form of the synthetic DNA-adaptor creates a new restriction site for BamHI allowing recloning of the entire con-struction. The clones pEB4, pEB5 and pEB9 containing transcriptional units of PTH with 4,5 and 9 bp spacer length were cloned into plasmids downstream from suitable promoters. The direct synthesis of fmet-hPTH 1-84 can then be expected in E.coli cells. Figure 2 summarizes the construction of the PTH transcriptional units.

```
                              GATCTGTG...PTH      Sau 3A fragment of p2C.36
                                  ACAC            dGTP, dATP fill-in, +S1
                                   ↓
                               TCTGTG...PTH
                                  AGACAC
                                   ↓             + CATCGATG - linker
                           CATCGATG.TCTGTG...PTH
                                                 + ClaI, subcloning
                                   ↓             + HindIII (B), fill-in,
                                                   + S1
                        TCATCG.ATGTCT...PTH
                       TTCATCG.ATGTCT...PTH
                      CTTCATCG.ATGTCT...PTH
                     GCTTCATCG.ATGTCT...PTH
                    AGCTTCATCG.ATGTCT...PTH
                                                 + RBS-linker, + BamHI
              GATCCCTAGGGAAGCTTCATCG.ATGTCT...PTH    transcriptional units
                    STOP  RBS
```
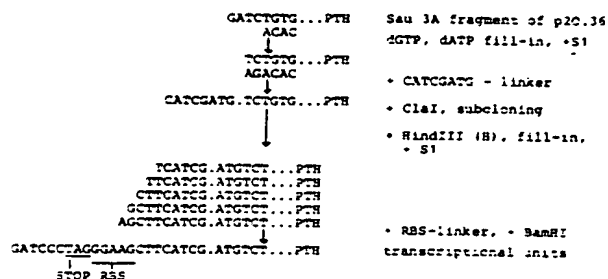
Fig. 2 shows the construction of the PTH transcriptional units which
is described in more detail in the text. The resulting transcriptio-
nal units consist of a BamHI-site at the 5'-end, a TAG-stop codon, a
RBS, a spacer of different lengths, an ATG initiation codon and the
full length PTH 1-84 gene.

For direct expression of hPTH 1-84 in E.coli transcriptional units of
PTH were brought under the control of lac, trp and tac - promoters.
The hybrid plasmids are named pPTHtac4, pPTHtac5, pPTHtac9, pPTHtrp4,
pPTHtrp5 and pPTHtrp9, pPTHT$_5$9.

Identification_of_hPTH_from_bacterial_extracts
Extracts of different E.coli strains containing the expression plas-
mids were tested for hPTH expression by direct radioimmunoassay
(Table 1). The amount of PTH under the control of the lac promoter is
comparable to the level of α-interferon under the control of the lac-
promoter (15). The amount of PTH under the control of trp-promoter is
1.4 times that produced under the lac 9-promoter. An E.coli host
SK2124 that contained mutations that increased the stability of euka-
ryotic mRNA for catabolic dehydroguinase was transformed with the re-
combinant plasmids but did not express higher levels of PTH. The ex-
pression level in hosts deficient in the capR (lon) protease was not
consistently higher than in the host HB101. An assessment of the sta-
bility of PTH at different growth phases was made. Cultures were grown
and chloramphenical (Cm (150/µg/ml)) was added to stop protein syn-
thesis (Fig. 3). The half-life of PTH at the log phase was 30 min., at
37°C, at stationary phase 15 min. This stability is comparable to that
of human proinsulin of 14 minutes (16).

Fig. 3: Ce
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anscriptional units which
.e resulting transcriptio-
-end, a TAG-stop codon, a
initiation codon and the

i transcriptional units of
trp and tac - promoters.
Htac5, pPTHtac9, pPTHtrp4,

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er levels of PTH. The ex-
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 An assessment of the sta-
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In Vivo-Stability of h PTH in E. coli
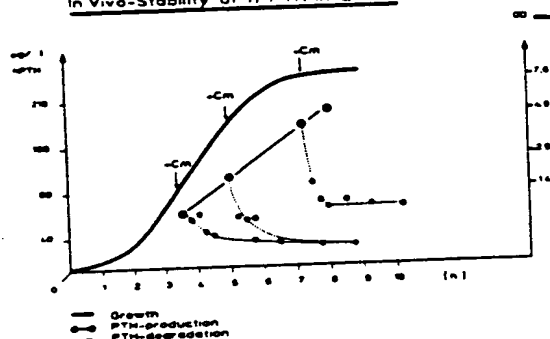
Fig. 3: Cells were grown in LB-Medium at 37°C and 150 µg/ml Cm was
added. Aliquots were tested by RIA.

Table 1

Plasmid (promoter)	Strain	Growing stage (OD550)	PTH mol/cell ind.	PTH mol/cell n.ind.	PTH (µg/l)
phPTH(trp)4	SG4121	2.4	1130	-	8
-"-	-"-	8.2	450	-	9
phPTH(trp)5	-"-	1.6	-	630	13
-"-	-"-	1.6	2140	-	44
-"-	-"-	3.8	-	630	41
-"-	-"-	2.5	2150	-	75
-"-	-"-	4.2	-	590	68
-"-	-"-	2.9	4450	-	189
-"-	-"-	4.3	-	476	16
-"-	-"-	4.3	781	-	59
phPTH(trp)9	-"-	1.7	-	4404	39
-"-	-"-	1.4	3296	-	41
-"-	-"-	3.8	-	3236	203
-"-	-"-	1.9	6790	-	207
-"-	-"-	4.1	-	427	30
-"-	-"-	2.4	4580	-	152
phPTH(lac)9	JM103	3.0	-	1458	65
-"-	-"-	2.8	3250	-	138
-"-	-"-	3.8	-	409	52
-"-	-"-	6.2	1117	-	142
-"-	-"-	8.8	-	132	30
-"-	-"-	8.8	150	-	38
-"-	SG4121	1.3	3250	-	11
-"-	-"-	8.8	820	-	18
phPTH(tac)4	-"-	1.4	1300	-	5
-"-	-"-	9.5	200	-	4
phPTH(tac)5	-"-	2.1	850	-	5
-"-	-"-	8.5	300	-	5
phPTH(tac)9	-"-	1.9	900	-	5
-"-	-"-	8.8	350	-	8

Expression of hPTH under the control of lac, trp, tac - promoters
with variable spacer length 4-9 between the RBS and ATG in different
hosts.

The immunological and biological reactivity of the PTH expressed in
E.coli was tested. The competition of 125-J hPTH by unlabelled pure
hPTH (standard curve) is compared to the competition behaviour of a
PTH extract from an E.coli carrying the plasmid phPTHT$_5$9. Both curves
are identical meaning that E.coli-synthesised-hPTH is immunologically
identical to pure hPTH. An extract of E.coli carrying the vector
plasmid pDS1 without PTH-gene sequences shows no competition under
identical conditions (Fig. 4).
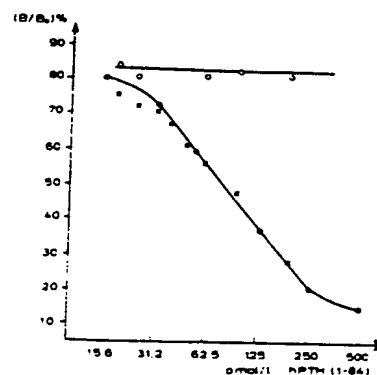
Fig. 4 compares the immunologi-
cal behaviour of the human PTH
(1-84) standard, the extract of
an E.coli SG4121 strain contain-
ing phPTHT$_5$9 plasmid which codes
for human PTH (1-84) and the ex-
tract of the same strain con-
taining the pDS1 vector plasmid
as negative control.
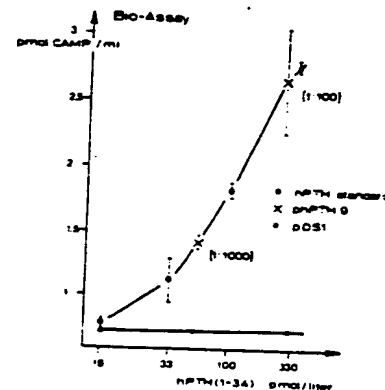
Fig. 5 shows the biological acti-
vity in the PTH specific renal
adenyl cyclase bioassay of E.coli
extracts containing the PTH ex-
pression plasmid phPTHT$_5$9 and the
pDS1 vector strain as negative
control.

The biological activity of the bacterially made hPTH was analysed in
vitro from phPTHT$_5$9 E.coli extracts by the adenyl-cyclase assay which
depends on the specific induction of adenyl cyclase through PTH con-
verting ATP to cAMP. The specific activity found was comparable to the
values found by radioimmunoassays. The E.coli carrying the control
plasmid was negative under the same conditions (Fig. 5).

Acknowledgements

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of the PTH expressed in
hPTH by unlabelled pure
petition behaviour of a
mid phPTHT$_5$9. Both curves
d-hPTH is immunologically
carrying the vector
s no competition under

Bio-Assay

[1.100]

● hPTH standard
x phPTH 9
● pos1

[1.1000]

33   100   300
hPTH(1-34) pmol/liter

ows the biological acti-
he PTH specific renal
clase bioassay of E.coli
containing the PTH ex-
plasmid phPTHT$_5$9 and the
or strain as negative

de hPTH was analysed in
enyl-cyclase assay which
yclase through PTH con-
und was comparable to the
carrying the control
s (Fig. 5).

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f this work.

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gene

an international journal focusing on gene cloning and gene structure and function

High-level production of human parathyroid hormone in *Bombyx mori* larvae and BmN cells using recombinant baculovirus

(PTH; cDNA; silkworm; cells; osteoblast function tests)

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SUMMARY

A full-length cDNA encoding human parathyroid hormone (hPTH) containing the prepro region was cloned into *Bombyx mori* baculovirus under the control of the polyhedrin promoter and polyadenylation sequences. After transfection and generation of the recombinant baculovirus, hPTH production was examined in silkworm larvae and BmN cell cultures. The larvae synthesized and efficiently secreted the correctly processed and authentic hPTH (9.4 kDa) with no sign of internal degradation. In BmN cells, the major secreted form was the correctly sized protein, but small amounts of degraded hPTH could also be detected in the medium by immunoblotting. Unlike the situation in larvae, prepro-hPTH could also be demonstrated intracellularly in BmN cells. The concentration of hPTH in the larval hemolymph was about 70 mg/l, as compared to approx. 55 μg/l in the medium per 7.5×10^6 cells. Recombinant hPTH (re-hPTH) from the hemolymph was purified by reverse-phase HPLC and subjected to chemical and biological analyses. The authenticity of the purified re-hPTH was confirmed by N-terminal sequencing, amino acid composition and a mass of 9425 Da, close to the theoretical value. The hormone showed high-affinity receptor binding and full biological potency in increasing cellular cAMP.

INTRODUCTION

Human parathyroid hormone (hPTH) is synthesized in the parathyroid glands as a prepro-hormone consisting of 115 aa. During processing, the pre and pro parts of the hormone are sequentially cleaved off resulting in the formation of the mature 84-aa hormone (Cohn and MacGregor, 1981). hPTH (1–84) is secreted in response

to a lowering of serum Ca^{2+} ions, and its physiological function is to elevate serum Ca^{2+} and to maintain the calcium and phosphate homoeostasis (Potts et al., 1982; Reeve et al., 1980). Prolonged and intermittent administration of low to medium doses of biologically active hPTH fragment has been shown to vigorously stimulate bone formation in animals and patients with osteoporosis (Reeve et al., 1980; 1991; Bradbeer et al., 1992).

Considering the potential pharmaceutical importance of the hormone in treatment of bone metabolic disorders, attempts have been made to produce the hormone employing different expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae* and mammalian cells (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Rokkones et al., 1994). The existence of internal protease sensitive domains has made hPTH susceptible to degradation and inactivation (Høgset et al., 1990; Gabrielsen et al., 1990). Thus, an optimalized system for expression of hPTH demands a correct processing without aberrant cleavage so that a high production efficiency can be obtained.

In this paper we compare the expression of hPTH in BmN cells and *Bombyx mori* (*Bm*) larvae using the *Bm* baculovirus with the polyhedrin promoter and regulatory sequences (Maeda, 1989a,b). The results demonstrate that the cells and the larvae are fully able to recognize the human signal and pro-part of hPTH.

RESULTS AND DISCUSSION

(a) Construction of recombinant virus for hPTH expression

Strategies followed for the cloning of full length hPTH cDNA (from pPPTH7) including its prepro part into the vector pBm030 is shown in the Fig. 1 and explained in legend. The re-vector pBmPTH84 harbours the full-length hPTH cDNA, including the human signal(pre) sequence and its pro part and is controlled by virus regulatory elements. Cotransfection of BmN cells in culture with the plasmid pBmPTH84 DNA and wt viral DNA (BmNPV) resulted in the formation of polyhedrin-negative re-plaques. Upstream and downstream from the cloned hPTH cDNA, about 3 kb viral flanking sequences are present, and during cotransfection, these flanking regions will facilitate homologous recombination so that the polyhedrin gene of the wt virus is replaced with the hPTH cDNA. After identification and isolation of re-viral plaques they were purified as described in Methods in the legend to Fig. 2. The re-virus were screened and those giving highest expression of hPTH were chosen for further experiments.

(b) Production and secretion of hPTH into larval hemolymph

Hemolymph samples from larvae infected with re-virus and collected after 24, 48 and 72 h, showed a time-dependent increase in two peptides (9.4 kDa and 14.3 kDa) which immunoreacted with hPTH antiserum, while hemolymph from wt virus-infected larvae was nega-
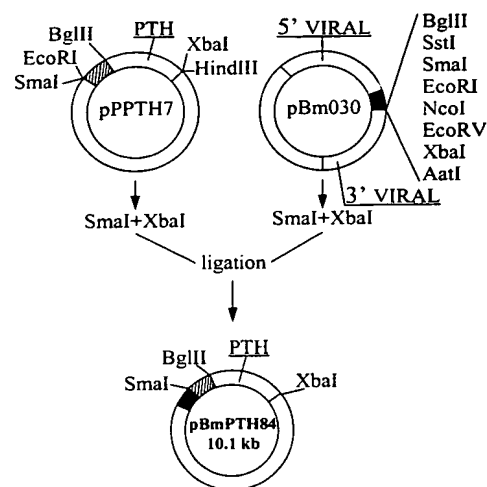
Fig. 1. Construction of baculovirus vector pBmPTH84. The entire hPTH cDNA signal and coding sequences were excised from the plasmid pPPTH7 by SmaI + XbaI digestion and ligated between the SmaI and XbaI sites of the vector pBm030 (Maeda, 1989b). In the resulting re-vector pBmPTH84 lacking the polyhedrin-encoding gene, the hPTH coding sequence is under the control of the polyhedrin promoter, transcriptional termination and polyadenylation sequences, but employing hPTH signal sequence. **Methods**: Plasmid DNA manipulations were performed essentially as described by Sambrook et al. (1989). A hPTH cDNA 413-bp fragment from plasmid pPPTH7 was ligated into vector pBm030, after cleavage with SmaI + XbaI, and transformed into the *E. coli* DH5α. The clones that contained the hPTH cDNA insert were identified and the re-vector named pBmPTH84. The junctions of hPTH cDNA and the transfer vector were confirmed by sequencing. Restriction enzymes and other DNA metabolizing enzymes were obtained from New England Biolabs. A anti-rabbit-[^{125}I]IgG was from Amersham. Synthetic hPTH (1–84) from Bachem was used as standard. All the other chemicals used were from Sigma.

tive (Fig. 2A, lanes 2, 3 and 4 versus 1). When standard hPTH(1–84) was loaded on the gel, it appeared as a 9.4-kDa form as expected (Fig. 2A, lane 6). However, when the same standard was mixed with the control-hemolymph, the same two different immunoreactive peptides appeared (9.4 kDa and 14.3 kDa) (Fig. 2A, lane 5). Thus, the 14.3-kDa band appeared to be a hPTH-binding protein as also confirmed in subsequent analysis. hPTH production increased during this period and the highest level was obtained after 72 h, whereafter the larvae succumbed to an infection. A semiquantitative estimation of hPTH in hemolymph collected the 3rd day of infection was carried out. Comparing the intensities of immunoreactivity to the different amounts of known hPTH standards as shown in Fig. 2B when different amounts of hemolymph sample was analyzed, it was estimated that 4 μl contained 0.25–0.5 μg hPTH (Fig. 2B, lanes 5, 6 and 7 versus lanes 2, 3 and 4). The non saturable binding properties of the 14.3-kDa band was verified by addition

A

B

kDa
25.7_

18.4_

14.3_
hPTH 9.4_
6.2_

3.0_

1 2 3 4 5 6

Fig. 2

C
kDa

25.7 _

18.4 _

14.3 _
hPTH 9.4_
6.2 _

3.0 _

1 2 3 4 5 6 7 8 9

19s_
18s_

1 2 3 4 5

kb

_1.77
_1.52
_1.28

_0.78

_0.40
_0.28

_0.16

1 2 3 4 5

Fig. 3

Fig. 2. The re-hPTH expression. (A) Time-course study of hPTH expression in hemolymph from infected larvae collected after 24, 48 and 72 h of infection with re-baculovirus (lanes 2, 3 and 4) compared to control represented by 72 h of wt infection (lane 1) using Western blot technique and mid-region specific anti-hPTH antibody. 3 μl per lane using 0.1% SDS-15% PAGE. Lane 5: hemolymph from wt infected larvae added 0.25 μg hPTH(1–84) standard. Lane 6: 0.25 μg hPTH(1–84) standard. (B) Semiquantitative estimation of hPTH produced in hemolymph 3 days after infection with wt (lane 1 (4 μl)) and re-baculovirus (lanes 5 (1 μl), 6 (2 μl) and 7 (4 μl)) and subjected to 0.1% SDS-15% PAGE, followed by immunoblot analysis. Lanes 2, 3 and 4: hPTH(1–84) marker (Bachem), 0.75 μg, 0.5 μg and 0.25 μg, respectively. Lanes 8 and 9: 4 μl of hemolymph from wt infected larvae added 0.5 μg and 0.25 μg hPTH(1–84) marker, respectively. (C) hPTH in BmN cell cultures 4 days after infection with re-virus (lane 2) and wt virus (lane 1). 5 ml of culture medium partly purified on a Sep-Pak column freeze-dried and analyzed by SDS–PAGE as in panel B. Lanes 3, 4 and 5: 0.05 μg, 0.1 μg and 0.075 μg, respectively, of hPTH(1–84) standard. **Methods:** Bm larva and BmN cell culture: The silkworm Bm larvae (TW × NB4D2) were fed ad libitum on fresh mulberry leaves and reared in the laboratory following the method of Krishnaswamy et al. (1973). BmN cells were grown in TC-100 medium containing 10% fetal calf serum and 50 μg gentamycin per ml at 27°C (Maeda, 1989a,b). Transfection and isolation of re-virus: Re-vector pBmPTH84 was amplified and purified. Subconfluent monolayers of BmN cells were co-transfected with purified infectious Bm wt baculovirus (BmNPV) DNA and the re-vector pBmPTH84. Homologous recombination between the plasmid DNA and wt viral DNA occurred in the Ca·phosphate mediated cotransfected cells as tested after 5 days by plaque assay, and the polyhedrin-negative plaques were screened for hPTH production in the BmN cells and Bm larvae. Collection of larval hemolymph, fatbody, BmN cell culture medium and cell lysate: Early fifth instar (24 h old) Bm larvae were needle inoculated with 50 μl of recombinant viral solution (3 × 10^5 pfu) into the body cavity using wt virus and saline injections as controls. Hemolymph was collected and treated as described (Maeda, 1989a). BmN cells (7.5 × 10^6 cells) were seeded in a tissue culture flask and after overnight incubation, the cells were infected by re-virus or wt virus (10 pfu/cell) separately. After 4 days of infection the medium was collected, the samples centrifuged at 1400 rpm for 5 min and the medium and cell pellet stored separately. All the samples were stored at −70°C till further analysis. Protein determination: Protein in hemolymph was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. PAGE and immunoblotting: 0.1% SDS-15% PAGE was performed under reducing conditions (Laemmli, 1970) and samples were solubilized as previously described (Towbin et al., 1979; Gabrielsen et al., 1990). Semiquantitative protein determination of hPTH: Semiquantitative measurements of hPTH using light scanning were performed on X-ray films developed after Western immunoblots using Bio Image system, Millipore.

Fig. 3. mRNA was prepared from fatbody cells and analyzed on agarose gels followed by Northern blot and hybridization to a hPTH cDNA fragment. Lanes: 1, 1.3 μg mRNA from wt-infected larvae (control) 72 h post-infection; 2, 3 and 4, 1.3 μg mRNA from re-virus-infected larvae 24, 48 and 72 h after infection; 5, 1 μg of PTH-mRNA isolated from human parathyroid adenomas. Ribosomal RNAs corresponding to 18S and 19S, respectively, are indicated. **Methods:** Total RNA was extracted from larval fatbody (wt virus infected and re-virus infected) as described (Glišin et al., 1974). Poly(A)+RNA was selected from identical amounts of total RNA from all the extractions using magnetic oligo(dT) Dynabeads (Dynal A.S. Norway). For time-course response, RNA was extracted at every 24 h post infection for a period of 3 days. The poly(A)+RNA samples were subjected to electrophoresis on a 1.5% agarose gel containing 6% (v/v) formaldehyde in 20 mM Na·phosphate pH 7.0 buffer. The RNA was subsequently transferred to a nylon membrane by passive diffusion and immobilized by UV light (2 min) and baked at 80°C for 1 h. The hPTH cDNA XbaI-EcoRI fragment was used for probe, and filters were subjected for hybridization at 42°C following standard procedures (Sambrook et al., 1989).

of 0.5 μg and 0.25 μg of hPTH, respectively, to the wt hemolymph (Fig. 2B, lanes 8 and 9) which by itself contained non-detectable immunoreactivity (Fig. 2B, lane 1).

(c) Production and secretion of hPTH by BmN cells into culture medium

BmN cells infected with re-virus also produced and secreted hPTH into the medium. Two secreted proteins were detected on immunoblots of SDS-PAGE using mid-

region specific anti-hPTH antiserum (Fig. 2C, lane 2). One comigrated with hPTH(1–84) standard (9.4 kDa) and the other and somewhat diffuse 5.5-kDa immunoreactive band probably represented proteolytic degradation products. The amount of hPTH was estimated to be about 0.3–0.5 μg from the flask with an initial concentration of 7.5 × 10^6 cells as determined by light scanning of the X-ray films developed after various times and compared to hPTH standards (lanes 3, 4 and 5). No immuno-

reactivity was found in medium from wt virus-infected cells (Fig. 2C, lane 1).

(d) The presence of h*PTH* mRNA

The presence of h*PTH* mRNA in fatbody cells was also studied 24, 48 and 72 h after infection and analyzed on agarose gels followed by Northern-blot and hybridization to a *PTH*cDNA *Xba*I-*Eco*RI fragment (Fig. 1) as probe. A time-dependent increase (about 100-fold) in transcripts corresponding in size to *PTH*mRNA prepared from human parathyroid adenomas was observed (Fig. 3, lanes 2, 3 and 4 versus lane 5). In addition, two higher M_r transcripts appeared of sizes equal to 18S and 19S rRNAs.

The presence of three mRNA species in the fat body may indicate heterogeneity within the non-translated regions since only one peptide form was demonstrated. They cannot be due to non-specific hybridization to remaining ribosomal RNA, since wt RNA gave no signal (Fig. 3, lane 1); in addition, a time-dependent increase was also observed.

(e) Intracellular hPTH in larvae and in cultured cells

Intracellular proteins from larval fatbody and BmN cells infected with re-virus were examined using immunoblots and compared to wt virus-infected controls. No hPTH immunoreactivity was detected in the larval fatbody cells while BmN cells showed two dominant hPTH immunoreactive bands. The major one of 13.5 kDa was similar to unprocessed prepro-hPTH while the 16-kDa protein could represent a modified variant or protein bound form of prepro-hPTH (data not shown).

(f) Quantitative measurements of hPTH by two-site chemiluminometric (sandwich) immunoassay

hPTH(1–84) in the hemolymph and culture medium was assayed using chemiluminometric immunoassay according to the manufacturer (Magic Lite, Ciba Corning, Germany). In hemolymph collected three days after coelomic infection, the hPTH concentrations were 0.05–0.1 g/l, while the total protein concentration was 63 g/l. In BmN cell culture medium at day 4 postinfection, the maximal hPTH concentrations were 40–70 µg/l per 10^6 cells.

(g) Reverse-phase HPLC-purification of hPTH and assessment of the chemical purity and authenticity

The re-hPTH was extracted from hemolymph and further purified as described in Methods to Fig. 4. The HPLC purification profiles are shown in Fig. 4 (A, B and C). hPTH from the last HPLC-step (Fig. 4C) was analyzed further on SDS-PAGE (Fig. 4D and E). The results from the gel analyses, including silver-staining (Fig. 4D) and immunoblot analysis (Fig. 4E) showed only one band with a mobility equal to standard hPTH and a purity equal to or better than the hPTH(1–84) Bachem standard (Fig. 4D and 4E, lanes 1 and 3 compared to lanes 2 and 4).

The purified PTH was also subjected to aa composition analysis and N-terminal sequencing which were consistent with the theoretical prediction (data not shown). Mass spectrometry was performed with a spectrum as shown in Fig. 5A and an M_r of 9425 was obtained from the single-charged molecular ions corresponding well
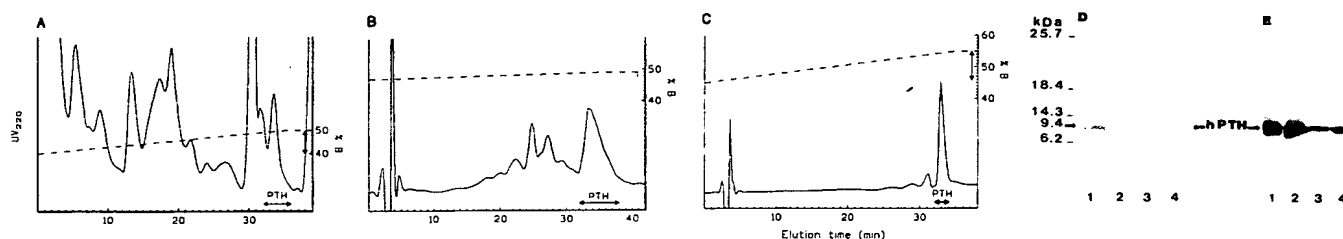
Fig. 4. Separation of re-hPTH(1–84) by reverse-phase HPLC from hemolymph of infected silkworms (A, B and C) and characterization by SDS-PAGE (D and E). (A) Preparative scale chromatography was performed on a Pharmacia SuperPac Pep-S C_{18}/C_2 column (22.5 × 250 mm) as previously described by Olstad et al. (1992) with small modifications. Eluant A. 0.115% TFA in MilliQ water; eluant B. 0.085% TFA in 70% aqueous acetonitrile (ACN) (10 ml/min). (B) Pooled fractions from A) containing hPTH were analyzed on an analytic Pharmacia SuperPac Pep-S C_{18}/C_2 column (4.0 × 250 mm) (linear gradient of 47–49% eluant B run for 35 min). Eluant A and eluant B were the same as in A. The flow rate was 1.0 ml/min. (C) Pooled fractions from B) containing hPTH were further purified on Pharmacia SuperPac Pep-S C_{18}/C_2 column (4.0 × 250 mm). A linear gradient of 45–55% eluant B was run for 35 min. Eluant A. 0.55% TFA in MilliQ water; eluant B. 0.45% TFA in 70% aqueous ACN. The flow rate was 1.0 ml/min. (D and E) 0.1% SDS-15% PAGE analysis of HPLC purified hPTH from hemolymph (Fig. 3C. fractions 33 and 34) and compared to hPTH(1–84) standard from Bachem. (D) Silver-staining. (E) Immunoblot analysis using the mid-region specific anti-hPTH antiserum. Lanes 1 and 3: 1.0 µg and 0.2 µg of hPTH from hemolymph. Lanes 2 and 4: 1.0 µg and 0.2 µg hPTH(1–84) standard (Bachem). Methods: *Purification of hPTH from the medium*: hPTH was concentrated and partly purified as described previously (Olstad et al. 1992) and after freeze-drying, the samples were dissolved in sample-buffer for SDS-PAGE analyses (Laemmli, 1970). *Reverse-phase HPLC*: Preparative and analytical scale chromatography was performed as described previously on SuperPac Pep-S C_{18}/C_2 column (22.5 × 250 mm/4.0 × 250 mm) (Reppe et al., 1991; Olstad et al., 1992). For silver staining of the gel, the procedure for the Sigma silver stain kit was followed.
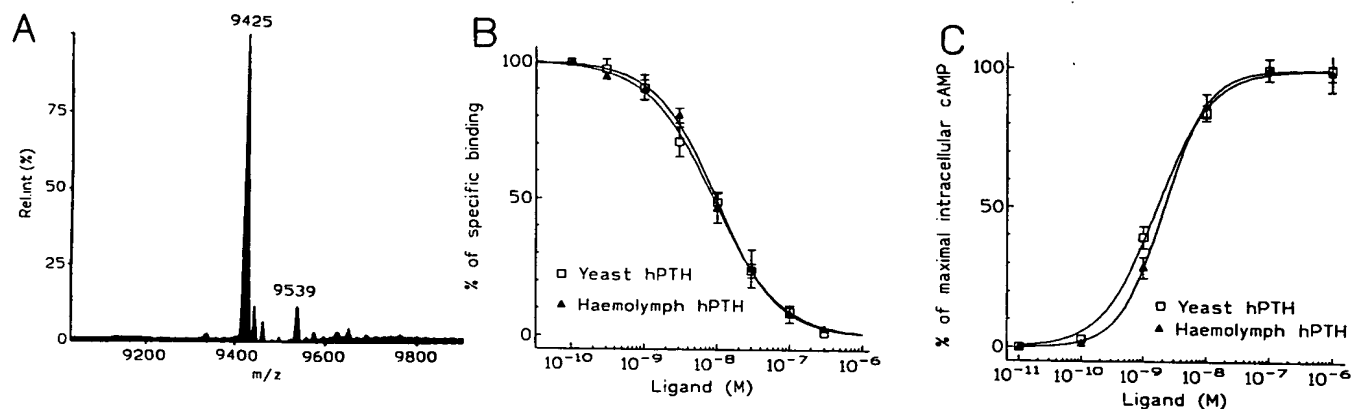
Fig. 5. Characterization of re-hPTH by mass-spectroscopy (A), radioreceptor binding (B) and intracellular cAMP stimulation (C). (A) Mass-spectroscopic analysis of recombinant hPTH(1–84). The dominant single peak represents a molecular hPTH mass of 9425 Da corresponding to the theoretical value of 9424.7 Da. (B) Inhibition of radiolabeled [Tyr^{36}]chicken-PTH-related protein(1–36)amide by different hPTHs. The re-hPTH(1–84) produced in infected silkworms and recombinant hPTH(1–84) (yeast hPTH) produced in Saccharomyces cerevisiae were tested in a radioreceptor assay using LLC-PK$_1$ cells transfected with the rat PTH/PTHrP· receptor. The data represent the mean±SEM of three independent experiments each performed in triplicate. (C) Stimulation of cellular cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor and stimulated (15 min, 37°C) with re-hPTH(1–84) produced in silkworms and re-hPTH(1–84) (yeast hPTH) produced in Saccharomyces cerevisiae is shown. The data represent the mean±SEM of three independent experiments each performed in duplicate. Methods: Mass spectrometry analysis was performed using a API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada) and carried out as described (Covey et al., 1988). Radioreceptor assay: LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993), were plated in 24-well plates (50000 cells/well) and grown for 2 days before incubation with ^{125}I-labelled [Tyr^{36}]chicken-PTHrP(1–36)NH$_2$ (100000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris·HCl, pH 7.7/100 mM NaCl/5 mM KCl/2 mM CaCl$_2$/5% heat-inactivated horse serum/0.5% heat-inactivated fetal calf serum) as described (Jüppner et al., 1988). The competing ligands were recombinant hPTH(1–84) expressed in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and recombinant hPTH(1–84) purified from hemolymph of infected silkworms. Techniques used for radioiodination of PTHrP analog were previously reported. Intracellular cAMP measurements. Measurements of intracellular cAMP in LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993) using Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min, then washed and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 M HCl. The stimulating ligands were re-hPTH(1–84) produced in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and re-hPTH(1–84) produced in Bm hemolymph.

with the theoretical M_r of 9424.7 for hPTH as calculated from the aa composition.

(h) Radioreceptor binding studies and intracellular cAMP measurements

Binding properties of two different re-hPTH forms from yeast and silkworm are shown in terms of displacement curves using the ^{125}I-labeled [Tyr^{36}]chicken-PTHrP(1–36)NH$_2$ as radioligand and LLC-PK$_1$ cells permanently transfected with the rat PTH/PTHrP receptor. Both hPTH forms representing the authentic hormones, showed equal receptor binding affinities (Fig. 5B) (calculated $K_d = 8.8 \pm 1.2 \times 10^{-9}$ M) and identical abilities to stimulate intracellular cAMP accumulation in the same cells with half maximal response obtained at 2.0×10^{-9} M (Fig. 5C).

(i) Concluding remarks and comparison of hPTH expression between various host systems

The quantitative result of re-hPTH production in BmN ovarian cell culture was much less than that in the hemolymph even corrected for the 30%–50% loss

during medium concentration and Sep-Pak column chromatography (see Methods in the legend to Fig. 4). The circulatory system of silkworm larvae opens into the coelomic cavity which is totally bathed in the hemolymph and retains the secretory proteins (Shigematsu, 1958).

hPTH could not be detected in the intracellular fractions of larval fatbody while the polypeptide and the assumed unprocessed forms were present in BmN cells. Ovarian cells are normally not designed for secretion of proteins, but rather for absorption, and this may be a reason for the low level of hPTH produced. The hormone and its mRNA were expressed in a parallel and a time-dependent fashion. The hPTH produced in the silkworm larvae was authentic as judged by N-terminal sequence, total aa composition and mass spectrometry. Also its receptor binding affinity and ability to activate the main second messenger system were identical to the yeast re-hPTH which previously was shown to have full biological activity in several target cell systems (Reppe et al., 1991).

The amount of hormone produced in the larval hemolymph represented 70 mg/l. This level was many times

higher than that reported for yeast (Gabrielsen et al., 1990; Reppe et al., 1991) and for *E. coli* (Høgset et al., 1990) secreted hPTH. The re-hPTH produced as an intracellular fusion protein with *S. aureus* protein A, showed a production after purification of 50–80 mg/l culture (Forsberg et al., 1991).

The production yield of hPTH in the silkworm larvae also compared well to ZZ-cecropin A fusion protein production in *Trichoplusia ni* larvae using *Autographa californica* baculovirus (Andersons et al., 1991). Production of human α-interferon in silkworm using *Bm* baculovirus, amounted to 30 mg/l hemolymph after purifying 10 ml of hemolymph by affinity column chromatography (Maeda et al., 1985). However, they did not report the concentration of α-interferon in the hemolymph prior to purification.

Expression of h*PTH* in microbiological systems has met with two problems related to incorrect N-terminal cleavage and aberrant intracellular processing (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Reppe et al., 1991; Forsberg et al., 1991; Rokkones et al., 1994). The major cleavage sites were after Lys^{26} in *S. cerevisiae* and after Val^{21} in *E. coli* (Rokkones et al., 1994). In mammalian cells, in contrast (mouse mammary tumor cell, i.e., C1271 cells, and Chinese hamster lung cells, i.e., DON cells) the entire h*PTH* cDNA including the prepro part gave rise to only the intact form (Rokkones et al., 1994). We demonstrate that the silkworm larvae in fact resembles the mammalian system cleaving the hPTH signal sequence correctly and that the human signal is also able to promote an efficient secretion of the intact hormone. However, the hPTH produced binds to a natural protein in the hemolymph and gives rise to a 14.3-kDa protein in addition to the expected 9.4-kDa form. The N-terminal sequence of the purified '14.3-kDa protein' was identical to hPTH(1–84) (data not shown). Standard hPTH also showed the same two bands when added to the hemolymph (Fig. 2A and B). Moreover, in buffer containing urea, the mobility of the 14.3-kDa form was normalized (data not shown) and also the 'acid treatment' occurring during HPLC purification released the peptide. Thus, the '14.3-kDa protein band' represents a hPTH-binding protein of unknown nature.

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CONTENTS (cont.)

GENE-COMBIS

CONTENTS

Hybrid gene synthesis: its application to the assembly of DNA sequences encoding the human parathyroid hormones and analogues[1]

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Bypassing any intermediate steps of purification and gene assembly, several synthetic oligonucleotides constituting a DNA duplex with a small base-mismatching region were phosphorylated, annealed, and ligated directly into a linearized plasmid vector. After transformation in bacteria, the two plasmid strands individualy yielded two different plasmids bearing altered versions of the same gene. Via this approach, DNA coding sequences of the human parathyroid hormone and analogues were synthesized and cloned in *Escherichia coli*.

Sung, W. L., Zahab, D. M., Yao, F.-L. & Tam, C. S. (1986) Hybrid gene synthesis: its application to the assembly of DNA sequences encoding the human parathyroid hormones and analogues. *Biochem. Cell Biol. 64*, 133–138

Evitant quelques étapes intermédiairies de purification et d'assemblage des gènes, nous avons phosphorylé, hybridé et lié directement dans un plasmide vecteur linéaire plusieurs oligonucléotides synthétiques, constituant un DNA bicaténaire comportant une petite région de mauvais appariement des bases. Après transformation dans les bactéries, les deux brins du plasmide produisent individuellement deux plasmides différents portant des versions altérées du même gène. Grâce à cette approche, nous avons synthétisé et cloné dans *Escherichia coli* les séquences de DNA codant pour l'hormone parathyroïdienne humaine et des analogues de cette hormone.

Introduction

PTH (1), a polypeptide of 84 amino acids (2), is an important regulator of calcium metabolism. Its stimulating effect on bone resorption by the osteoclast for the mobilization of calcium from bone to the extracellular fluid is well known (3); recent studies have shown that chronic PTH treatment can also result in an increase in bone density (4, 5). It is possible that this anabolic effect of PTH can be utilized to correct some bone-deficient states, like osteoporosis. However, this therapeutic potential has not yet been fully explored owing to the fact that sufficient quantities of this hormone at low cost are unavailable for clinical trial on a large scale.

The gene of the prepro-PTH was isolated from a human gene library (6). This gene encodes a peptide of 115 amino acids, which constitutes a precursor in the biosynthetic pathway of PTH. It was subsequently expressed in rat pituitary cells via a retrovirus (7). However, in our present study, we have adopted

chemical synthesis to prepare the human PTH gene for (*i*) the flexibility to select the frequently used yeast codons to facilitate expression in both *Escherichia coli* and the more discriminating yeast system; (*ii*) the addition of chemical cleavage sites (8), such as cysteine which is absent in PTH (6), at the terminals of PTH if it has to be expressed as a fused protein for additional stability; and (*iii*) the direct preparation of DNA sequences coding for pro-PTH, PTH, and PTH(1–34) with proven clinical effect on bone (5).

To reduce the work of oligonucleotide preparation, assembly, and cloning of these various versions of the PTH gene, we explored the possibility of hybrid gene synthesis. We envisage that a DNA heteroduplex with base mismatch in the gene-coding region can be prepared via ligation of synthetic oligonucleotides (Fig. 1). After insertion into a plasmid vector, the two plasmid strands act as individual templates for DNA replication (9–11) and will eventually yield two different plasmids bearing the two respective versions.

With this approach, we efficiently synthesized DNA sequences coding for the short fragments PTH(1–34) and PTH(1–40) and the PTH molecule with or without a cysteine codon substituting the termination codon.

Materials and methods

Enzymes and plasmid pUC8 were purchased from Bethesda
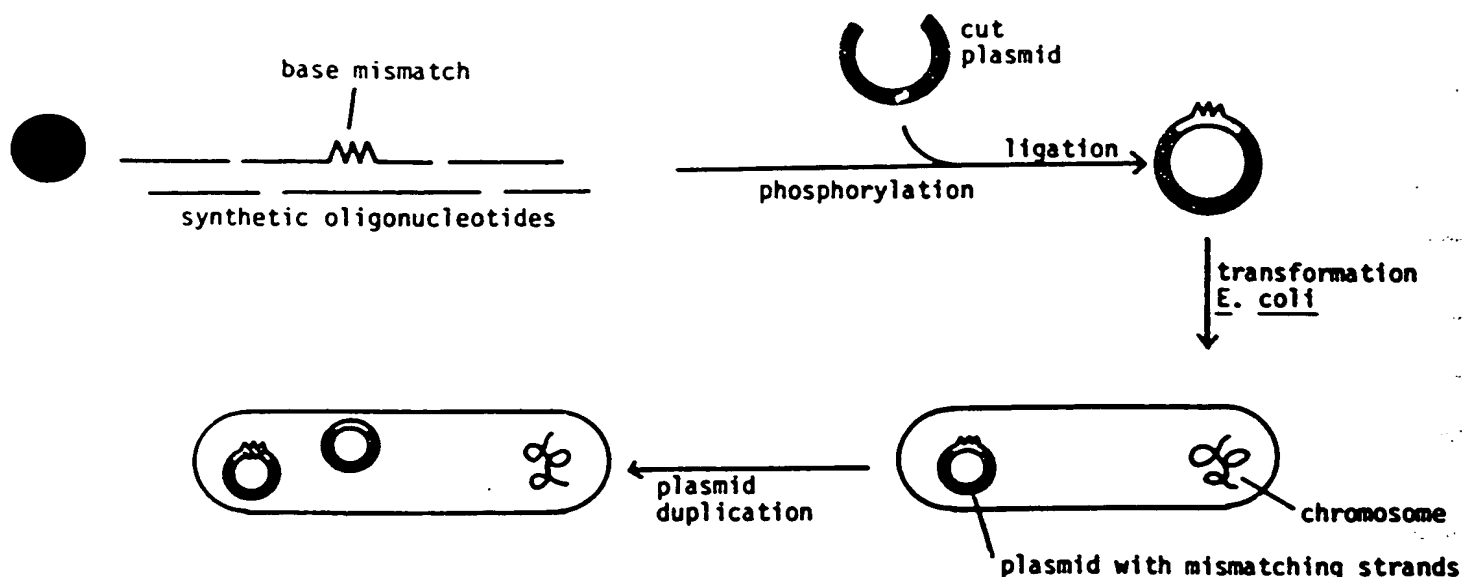
FIG. 1. Strategy for hybrid gene synthesis. Overlapping synthetic oligonucleotides with base mismatch can be phosphorylated, annealed, and ligated to linearized plasmid to yield heteroduplex. Inside bacterial host, the plasmid heteroduplex would yield two plasmid progenies, with both of its DNA strands as templates during replication.

chia coli K-12 strain JM 103 (Δ(*lac pro*), *thi*, *str A*, *sup E*, *end A*, *sbc B*, *hsd R*, F *tra* D36, *pro* AB, *lac I°*, ZΔ M15) was used in all cloning experiments.

Synthesis of ologonucleotides

The sixteen deoxyribooligonucleotides PI–PXVI (Fig. 2), encoding PTH with the frequently used yeast codons, were synthesized by DNA synthesizer model 380A (Applied Biosystem) and purified on 12% polyacrylamide gel containing 7 M urea.

Construction of plasmid pPTH-34 and pPTH-40

Each of the eight oligonucleotides PI–PVIII (1.3 pmol, 1 μL) (Fig. 2A) was phosphorylated in a mixture containing 0.4 μL of 10× kinase buffer, 0.4 μL of 1 mM ATP, 0.4 μL of T4 DNA kinase, and 3 μL of water. Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 3.5 μL of 10× ligase buffer, 3.5 μL of 4 mM ATP, 0.1 pmol of *EcoRI–HindIII* linearized plasmid pUC8, and 3.5 μL of T4 DNA ligase and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* JM 103 in YT plate containing ampicillin. Transformants were selected by the loss of β-galactosidase activity (X-Gal and isopropylthiogalactoside) for hybridization analysis.

Labelling of the hybridization probe

Oligonucleotides PI–PV (10 pmol, 1 μL) were phosphorylated individually with [³²P]ATP (10 pmol, 3 μL) in 1 μL of T4 DNA kinase, 1 μL of 10× kinase buffer, and 4 μL of water at 37°C for 1 h.

Screening of plasmid containing the PTH (1–40) gene

Colonies were chosen and grown on 10 copies of nitrocellulose filters on YT plates with ampicillin overnight. They were then denatured with 0.5 N NaOH – 1.5 M NaCl (10 min)

and neutralized with 0.5 N Tris–HCl (pH 7.0) – 1.5 M NaCl (10 min). After 2 h at 80°C in a vacuum oven, the filters were washed with 6× SSC – 0.05% Triton X-100 for 30 min. Cell debris was scraped off completely. After another 30 min in fresh solution, the duplicate filters were transferred individually into separate mixtures of 6× SSC – 1% dextran sulphate–0.05% Triton X-100 – 1× Denhardt's hybridization fluid. Individually appropriate ³²P-labelled probes were added to a pair of filters. After 16 h at 45°C, filters were washed twice with 6× SSC – 0.05% Triton X-100 at room temperature for 5 min and then at 45°C for 45 min, and were analyzed by autoradiography. Filters were washed again at 75°C for 45 min, followed by autoradiographic analysis.

Preparation of plasmids pPTH-34 and pPTH-40 via subcloning

Transformants positively identified by either probe PIV or PV were cultured for the minipreparation of plasmids to transform the *E. coli* JM 103 once again. Colony hybridization with ³²P-labelled probes PVI and PV were used to identify plasmid clones, pPTH-40 encoding the regular amino acid sequence from position 1 to 40 of PTH and pPTH-34 which has termination at oligonucleotide triplet position 35. The PTH-coding region was sequenced with the dideoxytermination method.

Construction of plasmids pPTH-84 and pPTH-87

Plasmid pPTH-40 was linearized by incubating with restriction enzymes *SstI* and *HindIII*. The phosphorylation of the other eight oligonucleotides PIX–PXVI (Fig. 2B) and their ligation into the linearized plasmid pPTH-40 were similar to the construction of the latter plasmid. Transformed JM 103, with plasmids bearing the whole PTH gene, was identified by hybridization with ³²P-labelled probes PIX–PXIII. Isolated plasmids were similarly subcloned and analyzed by DNA sequencing with the "dideoxy" method.

A

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                  1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18  19
                 SER VAL GLU ILE GLN LEU MET HIS ASN LEU GLY LYS HIS LEU ASN SER MET GLU
     EcoRI
5'-AA TTC TGT AAG GGA TCC AAG AGA TCG GTT TCT GAG ATC CAA TTG ATG CAT AAC TTG GGT AAG CAC TTG AAC TCT ATG GAA
    G   ACA TTC CCT AGG TTC TCT AGC CAA AGA CTC TAG GTT AAC TAC GTA TTG AAC CCA TTC GTG AAC TTG AGA TAC CTT
        PI                                                          PII
                        PVIII                                          PVII

20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39
ARG VAL GLU TRP LEU ARG LYS LYS LEU GLN ASP VAL HIS ASN PHE VAL ALA LEU GLY ALA
AGA GTT GAA TGG TTG AGA AAG AAG CTG CAG GAC GTT CAC AAC TTC | GTT | GCT TTG GGA GCT CCA TA
TCT CAA CTT ACC AAC TCT TTC TTC GAC GTC CTG CAA GTG TTG AAG | ATT | CGA AAC CCT CGA GGT ATT CGA-5'
    PIII                                              PIV                      TER    SstI    HindIII
                PVI                          PV
```

B

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40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64  65
PRO LEU ALA PRO ARG ASP ALA GLY SER GLN ARG PRO ARG LYS LYS GLU ASP ASN VAL LEU VAL GLU SER HIS GLU LYS
        SstI
        CCA TTG GCT CCA AGA GAC GCT GGT TCT CAA AGA CCA AGA AAG AAG GAA GAC AAC GTT TTG GTT GAA TCT CAC GAA AAG
3'-T CGA GGT AAC CGA GGT TCT CTG CGA CCA AGA GTT TCT GGT TCT TTC CTT CTG TTG CAA AAC CAA CTT AGA GTG CTT TTC
            PXVI                  PIX                              PX

66  67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84
SER LEU GLY GLU GLU ASP LYS ALA ASP VAL ASN VAL LEU THR LYS ALA LYS SER GLN TER
TCT TTG GGT GAA GCT GAC AAG GCT GAC GTT AAC GTG TTA ACT AAG GCT AAA TCG CAA | TAA | AGA TCT TGA
AGA AAC CCA CTT CGA CTG TTC CGA CTG CAA TTG CAC AAT TGA TTC CGA TTT AGC GTT | ACA | TCT AGA ACT TCG A-5'
    PXI                               PXIV                              PXII        BglII   HindIII
                                                              PXV                   CYS
                                                                                    PXIII
```

FIG. 2. Design of DNA sequences encoding PTH and analogues. Ends of oligonucleotides were indicated by arrows. Base-mismatching regions for generating analogues were contained in boxes. (A) Oligonucleotides PI–PVIII coding for PTH (positions 1–40) for the construction of plasmids pPTH-34 and pPTH-40. (B) Oligonucleotides PIX–PXVI coding for PTH (positions 38–84) for the construction of plasmids pPTH-84 and pPTH-87.
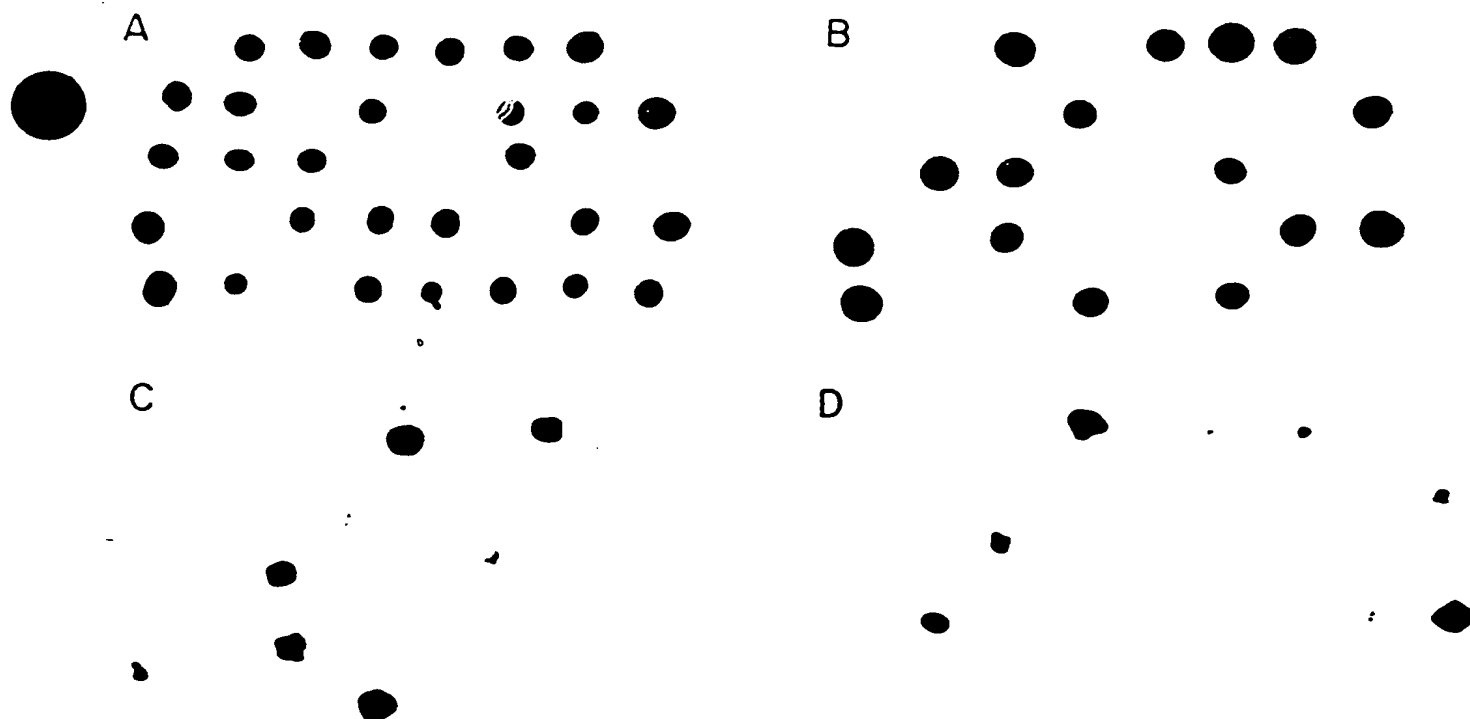
Fig. 3. After cloning of sequence encoding PTH(1–40), colony hybridization with the following. (A) ^{32}P-labelled probe PI, washed at 45°C. (B) PIV, washed at 45°C. (C) PIV, washed at 75°C, indicating plasmid progenies containing a valine codon at position 35 of the PTH sequence. (D) PV, washed at 75°C, indicating plasmid progenies containing termination codon at the same position.

Results

The eight synthetic oligonucleotides PI–PVIII, constituting the first half (oligonucleotide triplet positions 1–40) of the PTH gene (Fig. 2A), were phosphorylated and ligated directly into linearized plasmid pUC8 in a single operation without any intermediate purification or gene assembly.

Base mismatch was designed at nucleotide triplet position 35, between complementary oligonucleotides PIV (GTT, valine) and PV (TTA, complementary triplet of the termination codon) (Fig. 2A). Transformation in JM 103 by recombinant plasmid-bearing fragments PI–PVIII subsequently yielded two plasmids: pPTH-40 coding for a legitimate half of PTH (PTH 1–40) with the termination codon in the HindIII site and pPTH-34 encoding a shorter fragment (PTH(1–34)) because of the predetermined termination codon at triplet position 35.

Hybridization with ^{32}P-labelled PI–PV at 45°C identified transformants bearing the general PTH-coding sequence (Figs. 3A and 3B). At an elevated temperature of 75°C, both PIV and PV were capable of distinguishing between colonies predominant with plasmids pPTH-40 (Fig. 3C) and pPTH-34 (Fig. 3D), respectively. DNA sequencing of the two plasmids confirmed that pPTH-40

had a valine codon (complementary triplet AAC) at position 35 and pPTH-34 had termination (complementary triplet TTA) at the same site (Figs. 4A and 4B).

Plasmid pPTH-40 was then digested with restriction endonucleases SstI and HindIII. Synthesis of the whole PTH gene was then completed via phosphorylation and ligation of another eight synthetic oligonucleotides, PIX–PXVI constituting the rest of the PTH gene (positions 39–84) (Fig. 2B), into the linearized plasmid pPTH-40.

Base mismatch at oligonucleotide triplet position 85 of the two complementary fragments PXII (TAA, termination codon) and PXIII (ACA, complementary triplet of cysteine) resulted in the formation of two different PTH gene-bearing plasmids, pPTH-84 and pPTH-87. These bore the proper coding sequence with termination at position 85 or a cysteine codon (TGT) at the same position, respectively. The pPTH-87 plasmid with a newly created termination codon at position 88 and a unique BglII site at 86–87 would facilitate entry in the production of multimeric forms of PTH for additional stability.

Bacterial transformants with these plasmids were identified by hybridization with ^{32}P-labelled fragments PIX–PXIII as before. After subcloning of plasmids the
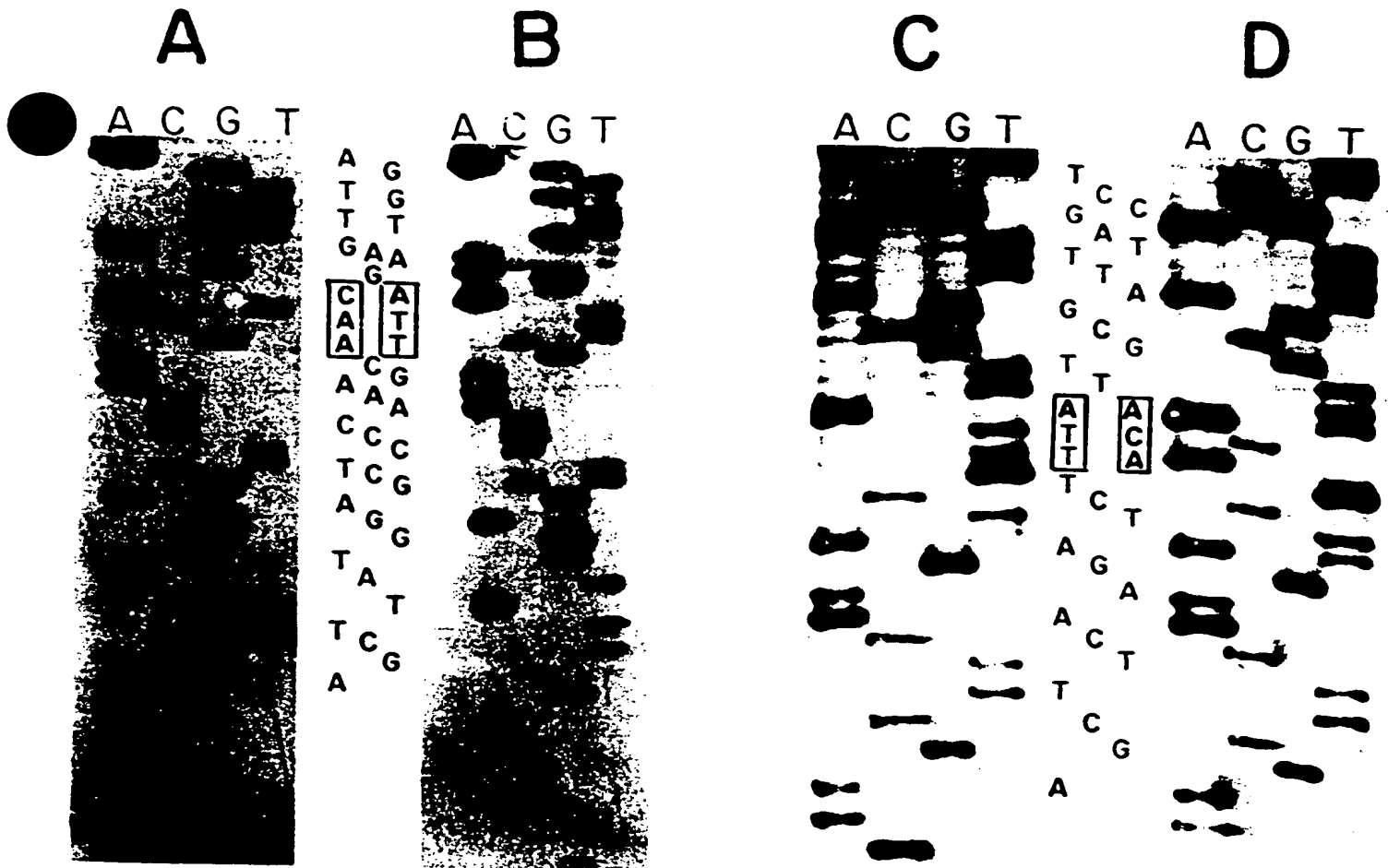
FIG. 4. DNA sequencing of PTH-coding region of different plasmid progenies, indicating modifications achieved (in boxes). Sequencing primer 5'-(ACG TTG TAA AAC GAC GGC) was used to yield the complementary sequence of PTH gene from the carboxyl terminus. (A) pPTH-40, AAC (valine) at position 35. (B) pPTH-34, TTA (termination) at the same position. (C) pPTH-84, TTA (termination) at position 85. (D) pPTH-87, ACA (cysteine) at the same position.

codons at position 85 of the two PTH-coding plasmids were further established by DNA sequencing, as termination (complementary triplet TTA) in pPTH-84 and cysteine (complementary triplet ACA) in pPTH-87 (Figs. 4C and 4D).

Discussion

Synthetic DNA duplex is generally designed with one strand coding for a single polypeptide and the other strand maintaining the duplex structure (12, 13). In our present design (Fig. 1), both DNA strands have been fully utilized to code for two related polypeptides. One strand codes for PTH; the other strand, with the appropriate base-mismatching region, codes indirectly other PTH analogues in the form of complementary sequences. Base mismatch in DNA duplex had also been used in the earlier development of the *in vitro* site-specific mutagenesis of bacteriophage and plasmid (9–11).

In our present procedure, each pair of oligonucleo-

tides which constitutes a part of the DNA duplex was constructed to leave two cohesive ends complementary to either the cohesive ends of the vector or to the adjacent pairs in the design. Therefore, ligation and cloning can be done in one single step, and at the same time the correct orientation with the plasmid vector and the sequence of the assembled genes can be ensured. Furthermore, the identification of transformants was made easy due to the loss of the cloning marker β-galactosidase in the plasmid vector. As shown by the results of hybridization, our method gives a much higher yield of mutant genes than the current techniques employed to produce a site-specific mutant. These advantages make our technique of hybrid gene synthesis a very attractive alternative to *in vitro* site-specific mutagenesis.

However, as demonstrated in the colony hybridization with probes PI and PIV (Figs. 3A and 3B), such a multifragment ligation can also generate some plasmid clones possessing only a partial gene-coding sequence,

In another unpublished experiment several overlapping fragments, without the terminal duplex for proper cloning, could still be inserted into plasmids. Therefore, is important to carry out hybridization with all fragments that constitute the cloned strands.

By deliberately introducing base mismatch into the complementary synthetic DNA strands, we have synthesized DNA sequences encoding human PTH and its analogues. Study is now conducted in the expression of these gene products.

With the recent success of the automated DNA synthesizer in preparing longer oligonucleotides than before, a greater degree of base mismatch or even multiple regions of base mismatch can probably be designed in the complementary strands of the heteroduplex without serious disruption of the duplex structure. We can therefore foresee that the present method can readily be extended to the construction of either closely related gene families or gene sequences encoding homologous polypeptides of different species.

Acknowledgements

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WPI Acc No: 84-295124/198448
XRAM Acc No: C84-125315
 **Hybrid vectors producing parathyroid hormone - which can be cloned in
pro- or eukaryotic cells**
Patent Assignee: GBF GES BIOTECH FORSCH (GBFB); GBF GES BIOTECH FORSCHUNG
   GMBH (GBFB)
Inventor: MAYER H
Number of Countries: 011 Number of Patents: 007
Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Main IPC	Week	
DE 3312928	A	19841122	DE 3312928	A	19830411		198448	B
DK 8401854	A	19841012					198503	
JP 60034986	A	19850222	JP 8471041	A	19840411		198514	
EP 139076	A	19850502	EP 84104063	A	19840411		198518	
DE 3312928	C	19870827					198734	
EP 139076	B1	19920701	EP 84104063	A	19840411	C12N-015/66	199227	
DE 3485797	G	19920806	DE 3485797	A	19840411	C12N-015/66	199233	
			EP 84104063	A	19840411			

Priority Applications (No Type Date): DE 3312928 A 19830411
Cited Patents: .11Jnl.Ref; A3...8720; EP 37723; EP 38182; EP 9930; No-SR.Pub
  ; WO 8401173; EP 9930; EP 37723; EP 38182
Patent Details:

Patent	Kind	Lan	Pg	Filing Notes	Application	Patent
DE 3312928	A		22			
EP 139076	A	G				

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Patent	Kind	Lan	Pg			
EP 139076	B1	G	17			

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Patent	Kind	Lan	Pg	Filing Notes		Patent
DE 3485797	G			Based on		EP 139076

Abstract (Basic): DE 3312928 A
      Hybrid vector which produces human parathyroid hormone (hPTH) and
   is closable in prokaryotic cells, comprising in sequence: a promoter; a
   DNA sequence of up to 1000 (esp. up to 200) b.p.; a ribosomal binding
   site; a DNA sequence of 4-15 bp; a start condon; and a DNA sequence
   (see Fig.1) coding for hPTH.
      Hybrid vector which produces hPTH and is closable in eukaryotic
   cells, characterised by: (a) being produced by isolating mRNA from pig
   parathyroid glands, cloning the mRNA as ds-cDNA in E.coli using a
   vector, isolating hybrid vector DNA from the clones, immobilising the
   DNA on a carrier, adding and then removing pig parathyroid mRNA,
   attempting to translate the mRNA into pig PTH, detecting any PTH formed
   to identify those clones having the pig PTH gene sequence, sequencing
   the hybrid vector DNA from those clones, identifying the clones having
   hybrid vector DNA with a sequence coding for pig PTH, radioactivity
   labelling hybrid vector DNA from the clones, using the labelled vector
   to screen a human gene bank, and converting the identified hPTH gene
   into a hybrid vector which is closable in eukaryotic cells; (b) having
   a specified DNA sequence (see Fig.2) or a subsequence thereof, between
   two RI cleavage sites.
Title Terms: HYBRID; VECTOR; PRODUCE; PARATHYROID; HORMONE; CAN; CLONE; PRO
  ; EUKARYOTIC; CELL
Derwent Class: B04; D16
International Patent Class (Main): C12N-015/66
International Patent Class (Additional): A61K-035/12; A61K-037/24;
  C07H-021/00; C12N-005/02; C12N-015/16; C12P-021/00
File Segment: CPI

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DIALOG(R)File 345:Inpadoc/Fam.& Legal Stat
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5527133
Basic Patent (No,Kind,Date): DK 8401854 A0 840409   <No. of Patents: 010>
Patent Family:

Patent No	Kind	Date	Applic No	Kind	Date	
AT 77834	E	920715	EP 84104063	A	840411	
DE 3312928	A1	841122	DE 3312928	A	830411	
DE 3485797	C0	920806	EP 84104063	A	840411	
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DK 8401854	A	841012	DK 841854	A	840410	
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EP 139076	A2	850502	EP 84104063	A	840411	
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Priority Data (No,Kind,Date):
    EP 84104063 A 840411
    DE 3312928 A 830411

PATENT FAMILY:
AUSTRIA (AT)
  Patent (No,Kind,Date): AT 77834 E   920715
    HUMAN-PARATHYROIDHORMON   (HUMAN-PTH)   PRODUZIERENDE   HYBRIDVEKTOR.
      (German)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH (DE)
    Author (Inventor): MAYER HUBERT DR
    Priority (No,Kind,Date):   EP 84104063   A   840411; DE 3312928 A
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    Applic (No,Kind,Date): EP 84104063 A   840411
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    IPC: * C12N-015/66; C12N-015/16
    CA Abstract No: * 102(09)073538R
    Derwent WPI Acc No: * C 84-295124
    Language of Document: German

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                                 EP 139076 P   920701
    AT 77834   R   950115   AT REN   CEASED DUE TO NON-PAYMENT OF THE
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                                 JAHRESGEB.)
GERMANY (DE)
  Patent (No,Kind,Date): DE 3312928 A1 841122
    HUMAN-PARATHORMON PRODUZIERENDE HYBRIDVEKTOREN UND HUMAN-PARATHORMONGEN
      (German)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH (DE)
    Author (Inventor): MAYER HUBERT DR (DE)
    Priority (No,Kind,Date): DE 3312928 A   830411
    Applic (No,Kind,Date): DE 3312928 A   830411
    IPC: * C12N-015/00; C12N-005/02; C12P-021/00; A61K-037/24; A61K-035/12
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    Language of Document: German
  Patent (No,Kind,Date): DE 3485797 C0 920806
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      (German)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH (DE)
    Author (Inventor): MAYER HUBERT DR (DE)
    Priority (No,Kind,Date): DE 3312928 A   830411
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IPC: * C12N-015/66; C12N-015/16
CA Abstract No: * 102(09)073538R
Derwent WPI Acc No: * C 84-295124
Language of Document: German
Patent (No,Kind,Date): DE 3312928 C2 870827
   HUMAN-PARATHORMON PRODUZIERENDE HYBRIDVEKTOREN, DEREN VERWENDUNG UND
      HUMAN-PARATHORMONGEN (German)
   Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
   Author (Inventor): MAYER HUBERT DR   (DE)
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   Applic (No,Kind,Date): DE 3312928 A   830411
   Filing Details: DE C2 D2 Grant of a patent after examination process
   IPC: * C12N-015/00; C12N-005/02; C12P-021/00; A61K-037/24; A61K-035/12
   Language of Document: German

GERMANY (DE)
   Legal Status (No,Type,Date,Code,Text):
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                                 (PATENTANMELDUNG))
                                 DE 3312928 A   830411
      DE 3312928   P   841122 DE A1   LAYING OPEN FOR PUBLIC
                                 INSPECTION (OFFENLEGUNG)
      DE 3312928   P   841122 DE OP8   REQUEST FOR EXAMINATION AS TO
                                 PARAGRAPH 44 PATENT LAW (PRUEFUNGSANTRAG
                                 GEM. PAR. 44 PATG. IST GESTELLT)
      DE 3312928   P   870827 DE D2   GRANT AFTER EXAMINATION
                                 (PATENTERTEILUNG NACH DURCHFUEHRUNG DES
                                 PRUEFUNGSVERFAHRENS)
      DE 3312928   P   880303 DE 8364   NO OPPOSITION DURING TERM OF
                                 OPPOSITION (EINSPRUCHSFRIST ABGELAUFEN OHNE
                                 DASS EINSPRUCH ERHOBEN WURDE)
      DE 3312928   P   950413 DE 8339   CEASED/NON-PAYMENT OF THE ANNUAL
                                 FEE (WEGEN NICHTZ. D. JAHRESGEB. ERLOSCHEN)
      DE 3485797   P   920806 DE REF   CORRESPONDS TO (ENTSPRICHT)
                                 EP 139076 P   920806
      DE 3485797   P   930729 DE 8364   NO OPPOSITION DURING TERM OF
                                 OPPOSITION (EINSPRUCHSFRIST ABGELAUFEN OHNE
                                 DASS EINSPRUCH ERHOBEN WURDE)
      DE 3485797   P   950413 DE 8339   CEASED/NON-PAYMENT OF THE ANNUAL
                                 FEE (WEGEN NICHTZ. D. JAHRESGEB. ERLOSCHEN)

DENMARK (DK)
   Patent (No,Kind,Date): DK 8401854 A   841012
   HYBRIDVEKTOR,   DER   PRODUCERER   HUMAN-PARATHYROID   HORMON,   SAMT
      HUMAN-PARATHYROID HORMON-GEN (Danish)
   Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
   Author (Inventor): MAYER HUBERT
   Priority (No,Kind,Date): DE 3312928 A   830411
   Applic (No,Kind,Date): DK 841854 A   840410
   IPC: * C07H; C12N
   Language of Document: Danish
   Patent (No,Kind,Date): DK 8401854 A0 840409
   HYBRIDVEKTOR,DER PRODUCERER HUMANPARATHYROID HORMON,SAMT HUMANPARATHYRO
      ID HORMON-GEN (Danish)
   Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
   Author (Inventor): MAYER HUBERT   (DE)
   Priority (No,Kind,Date): DE 3312928 A   830411
   Applic (No,Kind,Date): DK 841854 A   840410
   IPC: * C07H-021/
   Language of Document: Danish

DENMARK (DK)
   Legal Status (No,Type,Date,Code,Text):
      DK 841854   A   830411 DK AAA   PRIORITY OF THE APPL. (PATENT

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                          APPL.)
                          DE 3312928  A   830411
   DK 841854      A    840410  DK AEA        DATA OF DOMESTIC APPL.
                          DK 841854  A   840410
   DK 841854      A    841012  DK A          PUBLISHED APPLICATION
   DK 841854      A    941227  DK AHB        APPLICATION SHELVED DUE TO
                          NON-PAYMENT  (APPL. SHELVED DUE TO
                          NON-PAYMENT)
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EUROPEAN PATENT OFFICE (EP)
  Patent (No,Kind,Date): EP 139076 A2 850502
    HUMAN-PARATHYROID   HORMONE   (HUMAN-PTH)   PRODUCING   HYBRID   VECTORS,
      HUMAN-PARATHYROID HORMONE GENE, EUCARYOTIC CELLS CONTAINING THE
      HYBRID VECTOR AND THEIR USE (English)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
    Author (Inventor): MAYER HUBERT DR
    Priority (No,Kind,Date): DE 3312928 A   830411
    Applic (No,Kind,Date): EP 84104063 A   840411
    Designated States: (National) AT; BE; CH; DE; FR; GB; IT; LI; SE
    IPC: * C12N-015/00; C12P-021/02; C12N-005/00; C12R-001-91
    Language of Document: German
  Patent (No,Kind,Date): EP 139076 A3 870520
    HUMAN-PARATHYROID   HORMONE   (HUMAN-PTH)   PRODUCING   HYBRID   VECTORS,
      HUMAN-PARATHYROID HORMONE GENE, EUCARYOTIC CELLS CONTAINING THE
      HYBRID VECTOR AND THEIR USE (English)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
    Author (Inventor): MAYER HUBERT DR
    Priority (No,Kind,Date): DE 3312928 A   830411
    Applic (No,Kind,Date): EP 84104063 A   840411
    Designated States: (National) AT; BE; CH; DE; FR; GB; IT; LI; SE
    IPC: * C12N-015/00; C12P-021/02; C12N-005/00; C12R-001-91
    Language of Document: German
  Patent (No,Kind,Date): EP 139076 B1 920701
    HUMAN-PARATHYROID HORMONE (HUMAN-PTH) PRODUCING HYBRID VECTOR (English;
     French; German)
    Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH   (DE)
    Author (Inventor): MAYER HUBERT DR   (DE)
    Priority (No,Kind,Date): DE 3312928 A   830411
    Applic (No,Kind,Date): EP 84104063 A   840411
    Designated States: (National) AT; BE; CH; DE; FR; GB; IT; LI; SE
    IPC: * C12N-015/66; C12N-015/16
    CA Abstract No: * 102(09)073538R
    Derwent WPI Acc No: * C 84-295124
    Language of Document: German

EUROPEAN PATENT OFFICE (EP)
  Legal Status (No,Type,Date,Code,Text):
```
   EP 139076      P    830411  EP AA         PRIORITY (PATENT APPLICATION)
                          (PRIORITAET (PATENTANMELDUNG))
                          DE 3312928  A   830411
   EP 139076      P    840411  EP AE         EP-APPLICATION  (EUROPAEISCHE
                          ANMELDUNG)
                          EP 84104063  A   840411
   EP 139076      P    850502  EP AK         DESIGNATED CONTRACTING STATES
                          (BENANNTE VERTRAGSSTAATEN)
                          AT BE CH DE FR GB IT LI SE
   EP 139076      P    850502  EP A2         PUBLICATION OF APPLICATION
                          WITHOUT SEARCH REPORT   (VEROEFFENTLICHUNG DER
                          ANMELDUNG OHNE RECHERCHENBERICHT)
   EP 139076      P    870520  EP AK         DESIGNATED CONTRACTING STATES IN
                          A SEARCH REPORT   (IN EINEM RECHERCHENBERICHT
                          BENANNTE VERTRAGSSTAATEN)
                          AT BE CH DE FR GB IT LI SE
   EP 139076      P    870520  EP A3         SEPARATE PUBLICATION OF THE
                          SEARCH REPORT (ART. 93)   (GESONDERTE
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                              VEROEFFENTLICHUNG DES RECHERCHENBERICHTS
                              (ART. 93))
     EP 139076      P    871111   EP 17P        REQUEST FOR EXAMINATION FILED
                              (PRUEFUNGSANTRAG GESTELLT)
                              870916
     EP 139076      P    891004   EP 17Q        FIRST EXAMINATION REPORT
                              (ERSTER PRUEFUNGSBESCHEID)
                              890817
     EP 139076      P    920701   EP AK         DESIGNATED CONTRACTING STATES
                              MENTIONED IN A PATENT SPECIFICATION    (IN
                              EINER PATENTSCHRIFT ANGEFUEHRTE BENANNTE
                              VERTRAGSSTAATEN)
                              AT BE CH DE FR GB IT LI SE
     EP 139076      P    920701   EP B1         PATENT SPECIFICATION
                              (PATENTSCHRIFT)
     EP 139076      P    920701   EP REF        IN AUSTRIA REGISTERED AS:    (IN
                              AT EINGETRAGEN ALS:)
                              AT 77834   R    920715
     EP 139076      P    920806   EP REF        CORRESPONDS TO:    (ENTSPRICHT)
                              DE 3485797  P   920806
     EP 139076      P    920807   EP ET         FR: TRANSLATION FILED    (FR:
                              TRADUCTION A ETE REMISE)
     EP 139076      P    921104   EP GBT        GB: TRANSLATION OF EP PATENT
                              FILED (GB SECTION 77(6)(A)/1977)   (GB:
                              TRANSLATION OF EP PATENT FILED (GB SECT.
                              77(6)(A)/1977))
     EP 139076      P    930623   EP 26N        NO OPPOSITION FILED    (KEIN
                              EINSPRUCH EINGELEGT)
     EP 139076      P    941031   EP BERE       BE: LAPSED    (BE: BREVET REVOQUE)

                              940430 G.- FUR BIOTECHNOLOGISCHE FORSCHUNG
                              M.B.H. ;GBF
     EP 139076      P    941207   EP GBPC       GB: EUROPEAN PATENT CEASED
                              THROUGH NON-PAYMENT OF RENEWAL FEE
                              940411
     EP 139076      P    941230   CH PL/REG     PATENT CEASED
                              (LOESCHUNG/RADIATION/RADIAZION)
     EP 139076      P    950131   EP EUG        SE: EUROPEAN PATENT HAS LAPSED
                              (SE: EUROPEISKT PATENT HAR UPPHOERT ATT
                              GAELLA)
                              941110 84104063.7
     EP 139076      P    950224   FR ST/REG     LAPSED    (CONSTATATION DE
                              DECHEANCES)
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JAPAN (JP)
   Patent (No,Kind,Date):   JP 60034986 A2 850222
      HYBRID VECTOR FOR PRODUCING HUMAN PARATOLUMONE AND HUMAN PARATOLUMONE
         GENE (English)
      Patent Assignee: BIOTECHNOLOG FORSCHUNG GMBH
      Author (Inventor):   HIYUUBERUTO MEEYAA
      Priority (No,Kind,Date):   DE 3312928   A   830411
      Applic (No,Kind,Date):   JP 8471041   A   840411
      IPC: * C07H-021/04; A61K-035/12; A61K-035/30; A61K-035/74; A61K-037/24
         ; C12N-005/00; C12N-015/00; C12R-001-91
      Language of Document: Japanese

0139076

Europäisches
Patentamt

EUROPÄISCHER TEILRECHERCHENBERICHT,
der nach Regel 45 des Europäischen Patent-
übereinkommens für das weitere Verfahren als
europäischer Recherchenbericht gilt

Nummer der Anmeldung

EP 84 10 4063

EINSCHLÄGIGE DOKUMENTE

Kategorie	Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile	Betrifft Anspruch	KLASSIFIKATION DER ANMELDUNG (Int. Cl.3)
O,X	EXPERIENTIA, Band 39, Nr. 6, Juni 1983, Seite 659; Birkhäuser Verlag, Basel, CH W. BORN et al.: "Expression and processing of human preproparathyroid hormone in Escherichia coli." * Insgesamt *	1,2,4	C 12 N 15/00 C 12 P 21/02 C 12 N 5/00// (C 12 N 5/00 C 12 R 1/91) (C 12 P 21/02 C 12 R 1/91)
Y		3,5-8	

X	CHEMICAL ABSTRACTS, Band 94, Nr. 9, 2. März 1981, Seite 477, Ref.Nr. 62752w; Columbus, Ohio, US H.M. KRONENBERG et al.: "Cloning bovine and human parathyroid hormone DNA in bacteria." & ENDOCRINOL. PROC. INT. CONGR. ENDOCRINOL., 6th 1980, 508-11. * Zusammenfassung *	9	
Y		1-8,10	RECHERCHIERTE SACHGEBIETE (Int. Cl.3)
	---		C 12 N C 12 P
X	EP-A-0 038 182 (HARVARD COLLEGE)		

UNVOLLSTÄNDIGE RECHERCHE ./.,

Nach Auffassung der Recherchenabteilung entspricht die vorliegende europäische Patentanmeldung den Vorschriften des Europäischen Patentübereinkommens so wenig, daß es nicht möglich ist, auf der Grundlage einiger Patentansprüche sinnvolle Ermittlungen über den Stand der Technik durchzuführen.

Vollständig recherchierte Patentansprüche: 1-10

Unvollständig recherchierte Patentansprüche:

Nicht recherchierte Patentansprüche: 11

Grund für die Beschränkung der Recherche:

Verfahren zur chirurgischen oder therapeutischen Behandlung des menschlichen oder tierischen Körpers (Siehe Art. 52(4) des Europäischen Patentübereinkommens).

Recherchenort	Abschlußdatum der Recherche	Prüfer
Den Haag	13-02-1987	MADDOX

Europäisches
Patentamt   EUROPÄISCHER TEILRECHERCHENBERICHT

EINSCHLÄGIGE DOKUMENTE		KLASSIFIKATION DER ANMELDUNG (Int. Cl.3)
Kategorie	Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile	betrifft Anspruch	
	* Ansprüche 1,6,11,14 *	1-4	

Y	EP-A-0 009 930 (LELAND STANFORD UNIVERSITY)		
	* Seite 4, Zeilen 9-15, Zeile 32 - Seite 5, Zeile 3; Seite 6, Zeilen 4-11; Seite 7, Zeilen 4-35; Seite 9, Zeile 5 - Seite 10, Zeile 9, Zeile 33 - Seite 11, Zeile 9 *	1-4	

Y	EP-A-0 037 723 (UNIVERSITY OF CALIFORNIA)		RECHERCHIERTE SACHGEBIETE (Int. Cl.3)
	* Seite 3, Zeilen 8-21; Seite 6, Zeilen 35-38; Seite 7, Zeilen 33-38 *	5-8,10	

P,X	WO-A-84 01 173 (IMMUNO NUCLEAR CORP.)		
	* Seiten 17-21 *	7	

P,X	CHEMICAL ABSTRACTS, Band 100, Nr. 3, 16. Januar 1984, Seite 148, Ref.Nr. 18646h; Columbus, Ohio, US H. MAYER et al.: "Assignment of the human parathyroid hormone gene to chromosome 11." & HUM. GENET. 1983, 64(3), 283-5.		
	* Zusammenfassung *	9	

P,X	PROC. NATL. ACAD. SCI. USA, Band 80, April 1983, Seiten 2127-2131; T.J. VASICEK et al.: "Nucleotide sequence of the human parathyroid hormone gene."		
	* Insgesamt *	9	

A	CHEMICAL ABSTRACTS, Band 93, Nr. 5, 4. August 1980, Seite 339, Ref.Nr. 39767a; Columbus, Ohio, US G. WIDERA et al.: "Characterization of porcine parathyroid hormone mRNA, cDNA and cDNA-pBr322 hybrid plasmids."		

⑲ Europäisches Patentamt

European Patent Office

Office européen des brevets

⑪ Veröffentlichungsnummer: **0 139 076**

A2

⑫ **EUROPÄISCHE PATENTANMELDUNG**

㉑ Anmeldenummer: **84104063.7**

㉒ Anmeldetag: **11.04.84**

㊿ Int. Cl.⁴: **C 12 N 15/00**
C 12 P 21/02, C 12 N 5/00
//(C12N5/00, C12R1:91),
(C12P21/02, C12R1:91)

㉚ Priorität: **11.04.83 DE 3312928**

㊸ Veröffentlichungstag der Anmeldung:
02.05.85 Patentblatt 85/18

㉞ Benannte Vertragsstaaten:
AT BE CH DE FR GB IT LI SE

⑦ Anmelder: **Gesellschaft für Biotechnologische Forschung mbH (GBF)**
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⑭ Vertreter: **Boeters, Hans Dietrich, Dr. et al,**
Boeters, Bauer & Partner Thomas-Wimmer-Ring 14
D-8000 München 22(DE)

㉤ Human-Parathyroidhormon (Human-PTH) produzierende Hybridvektoren, Human-Parathyroidhormongen, eukaryotische Zellen mit dem Hybridvektor und deren Verwendung.

㊲ Die Erfindung betrifft Human-Parathormon produzieren-
de Hybridvektoren sowie Human-Parathormongen.

EP 0 139 076 A2

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PATENTANWÄLTE
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0139076

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DIPL.-ING. VINCENZ v. RAFFAY
DIPL.-CHEM. DR. THOMAS FLECK
HAMBURG

TELEFON: (089) 22 78 87
TELEX: 5 24 878 mm
TELEGRAMME: PROVENTION, MÜNCHEN

11. April 1983

Anmelderin: Gesellschaft für Biotechnologische
Forschung mbH (GBF), Mascheroder Weg 1,
D-3300 Braunschweig-Stöckheim

Human-Parathormon produzierende Hybridvektoren und Human-
Parathormongen

Human-Parathormon reguliert u.a. den Einbau und Ausbau von
5 Calcium in Knochen.

Aufgabe der Erfindung ist es, biologisches Material zur
Verfügung zu stellen, mit dem Human-Parathormon technisch
hergestellt werden kann.
10

Gemäß einer Ausführungsform wird diese Aufgabe durch einen
in prokaryotischen Zellen klonierbaren Hybridvektor gelöst,
der Human-Parathormon produziert und durch folgende Merk-
male gekennzeichnet ist:
15

(a) einen Promotor,

(b) einen sich an den Promotor anschließenden DNA-Bereich
von 0 bis 1000 und insbesondere 0 bis 200 Basenpaaren,

20 (c) eine sich an den DNA-Bereich gemäß (b) anschließende
ribosomale Bindungsstelle,

- 2 -

(d) einen sich an die ribosomale Bindungsstelle anschließen-
den DNA-Bereich von 4 bis 15 Basenpaaren,

(e) ein sich an den DNA-Bereich gemäß (d) anschließendes
Startcodon und

(f) die folgende Human-Parathormon kodierende DNA-Sequenz:

```
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His
TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT
AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG GAC CCT TTT GTA


Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu
CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG
GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA TTC TTC GAC


Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro Leu Ala
CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT
GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA GAT CGA


Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu Asp
CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA GAC
GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT CTG


Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT


Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG T
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC A
```

Als prokaryotische Zellen kommen alle Zellen in Betracht, in denen sich Hybridvektoren mit den angegebenen Merkmalen in technischem Maßstab unter Bildung von Human-Parathormon klonieren lassen. Insbesondere kommt Escherichia coli in Be-
5 tracht. Beispiele für geeignete Promotoren für E. coli finden sich beispielsweise bei Sengbusch, P. von, Molekular- und Zellbiologie, Springer-Verlag, Heidelberg etc. 1979. Bei E. coli kann die ribosomale Bindungsstelle beispielsweise die folgende DNA-Sequenz aufweisen:
10

AGGA oder GGAG
TCCT   CCTC

Ein Beispiel für ein bei E. coli verwendbares Startcodon
15 hat die DNA-Sequenz

ATG
TAC

20 Gemäß einer weiteren Ausführungsform wird die der Erfindung zugrundeliegende Aufgabe durch einen in eukaryotischen Zellen klonierbaren und Human-Parathormon produzierenden Hybrid- vektor gelöst,

25 (A) der dadurch herstellbar ist, daß man

(a) aus Schweine-Nebenschilddrüsen mRNA isoliert,

(b) die isolierte mRNA als ds-cDNA mit Hilfe eines Vektors in E. coli kloniert,

30 (c) aus Pools erhaltener Klone Hybridvektor-DNA isoliert,

(d) isolierte Hybridvektor-DNA an einen für jeden Pool eige- nen Träger fixiert, gemäß (a) isolierte mRNA anlagert und wieder entfernt, entfernte mRNA in Schweine-Prä-Pro-Parathor- mon oder Schweine-Parathormon zu übersetzen versucht, gebil- detes Schweine-Prä-Pro-Parathormon oder Schweine-Parathormon

- 5 -

durch Antikörperfällung nachweist und damit gemäß (b) erhaltene Klone ermittelt, die Schweine-Parathormongen-Sequenzen aufweisen, Hybridvektor-DNA ermittelter und Schweine-Parathormongen-Sequenzen aufweisende Klone sequenziert und den oder
5 die Klone identifiziert, die Hybridvektor-DNA mit Schweine-Parathormon kodierender DNA-Sequenz aufweisen,

(e) Hybridvektor-DNA der gemäß (d) identifizierten Klone radioaktiv markiert,

10 (f) mit erhaltener radioaktiv markierter Hybridvektor-DNA eine Human-Genbank screent und

(g) das ermittelte Human-Parathormon in einen in eukaryotischen Zellen klonierbaren Hybridvektor überführt,

(B) und der dadurch gekennzeichnet ist, daß er eine zwischen
15 zwei RI-Schnittstellen liegende DNA-Sequenz, die die folgende DNA-Sequenz umfaßt, oder einen Unterbereich der zwischen den RI-Schnittstellen liegenden DNA-Sequenz aufweist:

cannot be expressed in P. Cere.
since it requires correct splicing

TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

```
CATTGTAT|
GTAACATA|_____
                                 Intron II ca. 400 bp
```

```
                     Met Ile Pro Ala Lys Asp Met Ala Lys Val Met
         GTG .AAG |ATG ATA CCT GCA AAA GAC ATG GCT AAA GTT ATG
---------- CAC TTC|TAC TAT GGA CGT TTT CTG TAC CGA TTT CAA TAC
```

```
Ile Val Met Leu Ala Ile Cys Phe Leu Thr Lys Ser Asp Gly Lys
ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA AAA TCG GAT GGG AAA
TAA CAG TAC AAC CGT TAA ACA AAA GAA TGT TTT AGC CTA CCC TTT
```

```
Ser Val Lys|
TCT GTT AAG|
AGA CAA TTC|_____
```

```
Intron I    |Lys Arg Ser Val Ser Glu Ila Gln Leu Met His Asn
ca. 30 bp __|AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
            |TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG
```

```
Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg
CTG GGA AAA CAT CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT
GAC CCT TTT GTA GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA
```

```
Lys Lys Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro
AAG AAG CTG CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT
TTC TTC GAC GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA
```

```
Leu Ala Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu
CTA GCT CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA
GAT CGA GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT
```

```
Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
CTG TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT
```

```
Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln·
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG TGA
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC ACT
```

```
AAA TGA AAA CAG ATA TTG TCA GAG TTC TGC TCT AGA CAG TGT AGG
TTT ACT TTT GTC TAT AAC AGT CTC AAG ACG AGA TCT GTC ACA TCC
```

```
GCA ACA ATA CAT GCT GCT AAT TCA AAG CTC TAT TAA GAT TTC CAA
CGT TGT TAT GTA CGA CGA TTA AGT TTC GAG ATA ATT CTA AAG GTT
```

```
GTG CCA ATA TTT CTG ATA TAA CAA ACT ACA TGT AAT CCA TCA CTA
CAC GGT TAT AAA GAC TAT ATT GTT TGA TGT ACA TTA GGT AGT GAT
```

```
GCC ATG ATA ACT GCA ATT TTA ATT GAT TAT TCT GAT TCC ACT TTT
CGG TAC TAT TGA CGT TAA AAT TAA CTA ATA AGA CTA AGG TGA AAA
```

```
ATT CAT TTG AGT TAT TTT AAT TAT CTT TTC TAT TGT TTA TTC TTT
TAA GTA AAC TCA ATA AAA TTA ATA GAA AAG ATA ACA AAT AAG AAA
```

```
TTA AAG TAT GTT ATT GCA TAA TTT ATA AAA GAA TAA AAT TCG ACT
AAT TTC ATA CAA TAA CGT ATT AAA TAT TTT CTT ATT TTA AGC TGA
```

```
TTT AAA CCT CTC TTC TAC CTT AAA ATG TAA AAC AAA AAT GTA ATG
AAA TTT GGA GAG AAG ATG GAA TTT TAC ATT TTG TTT TTA CAT TAC
```

```
ATC ATA AGT CTA AAT AAA TGA AGT ATT CTT CAC TCA AA
TAG TAT TCA GAT TTA TTT ACT TCA TAA AGA GTG AGT TT
```

----- Prä-Pro   ———— PTH   - 6 -

Beispiele für eukaryotische Zellen, in denen der Hybridvektor
klonierbar ist und Human-Parathormon produzieren kann, sind
Humanzellen oder Affenzellen, beispielsweise Affennieren-
zellen.

5

Schließlich wird die der Erfindung zugrundeliegende Aufgabe
durch Human-Parathormongen gelöst,

(a) das gemäß den vorstehenden Ausführungen und Anspruch 3
(A) herstellbar ist und

10

(b) durch die zwischen zwei RI-Schnittstellen liegende DNA-
Sequenz gemäß den vorstehenden Ausführungen und gemäß An-
spruch 3 (B) oder einen Unterbereich davon gekennzeichnet
ist.

15

Zur Produktion von Human-Parathormon ist es auch möglich,
das erfindungsgemäße Human-Parathormongen direkt in eukaryo-
tische Zellen zu transformieren.

20 Nachstehend wird die Herstellbarkeit der erfindungsgemäßen
Hybridvektoren und des erfindungsgemäßen Human-Parathormon-
gens an einem Beispiel und drei Schemata näher erläutert.

Aus frisch geschlachteten Schweinen wurden die Nebenschild-
25 drüsen operiert. Aus diesem Drüsengewebe wurde mRNA isoliert.
Die isolierte mRNA wurde als doppelstrangige komplementäre
DNA (ds-cDNA) mit Hilfe eines Plasmids in E. coli kloniert.
Aus den erhaltenen Hybridklonen wurde die Hybridplasmid-DNA
isoliert. Aus den Nebenschilddrüsen isolierte mRNA wurde in
30 einem In-Vitro-Translationssystem in das Schweine-Prä-Pro-
Parathormon oder Schweine-Parathormon übersetzt. Das Schwei-
ne-Prä-Pro-Parathormon oder Schweine-Parathormon wurde durch
Antikörperfällung nachgewiesen. Mit Hilfe einer "Hybrid-
Arrested-Translation" konnten die Klone aufgefunden werden,
35 die Schweine-Parathormonsequenzen enthielten. Aus den ermit-

telten Hybridklonen wurde Hybridplasmid-DNA, die das Schweine-Parathormongen (Schema 1) umfaßte, radioaktiv markiert (nick-translatiert) und zum Screenen von Humangenbänken verwendet. Auf diese Weise ermitteltes Human-Parathormongen

5 wurde durch Subklonieren in einem Plasmid angereichert. Ein auf diese Weise angereichertes Human-Parathormongen wurde sequenziert. Die Sequenz des für die Expression von Human-Parathormon relevanten Abschnittes ist dem Schema 2 zu entnehmen. Die ermittelte Sequenz stimmte mit der bekannten cDNA-

10 Sequenz und mit der bekannten Aminosäuresequenz des Human-Parathormons überein.

Im folgenden wird Schema 3 erläutert. (1) In das weitere Verfahren wurde die zwischen zwei RI-Schnittstellen liegende

15 DNA-Sequenz eingesetzt, die die DNA-Sequenz des Schemas 2 umfaßt. (2) Es wurde nun mit Hilfe der Restriktionsendonuklease Sau3A das Prä-Pro-PTH-Gen (PTH = Parathormon) vom Gen des reifen 1-84-PTH getrennt. (3) Durch Auffüllen von dATP und dGTP mit dem "Large fragment" der E.-coli-DNA-Polymera-

20 se-I und (4) anschließenden Abbau mit S1-Nuklease wurde der verbleibende Einzelstrangrest (GA) der klebrigen Enden (sticky ends) beseitigt, die vom Sau3A-Schnitt herrührten (GATC); dadurch wurde das Codon "TCT" für die Aminosäure 1 (Serin) des Human-PTH rekonstituiert. (5) An dieses in der

25 angegebenen Weise behandelte PTH-DNA-Fragment wurde ein DNA-Adaptor ligiert. Dadurch wurde dem Serin ein Methionin-Codon "ATG" direkt vorgeschaltet. Dieses Codon ist eines der wichtigen Signale für den Start der Synthese von PTH im Mikroorganismus. (6-7) Das in der angegebenen Weise konstru-

30 ierte PTH-Fragment wurde in die ClaI-Spaltstelle von pBR322 subkloniert. Ausgewählt wurde ein Klon, dessen PTH-Gen vor der HindIII-Spaltstelle des pBR322 im Uhrzeigersinnorientiert war. Dieser PTH-Klon wurde mit HindIII gespalten. (8) Die "sticky ends" dieser Spaltstelle ließen Auffüllreaktionen mit

35

vier verschiedenen Nukleotiden zu. In Kombination mit dem
Abbau durch S1-Nuklease gelangte man zu Fragmenten mit aufge-
füllten Enden (flush ends), deren Entfernung vom ATG-Codon 4
bis 10 Basenpaare betrug. (9) Vor diese Variation an Frag-
5 menten wurden zwei synthetische DNA-Adaptoren ligiert, und
zwar /TCCCTAGGGA/+/TCCCTAGGGA/. Diese Linker enthielten die
Sequenz der ribosomalen Bindungsstelle, ein weiteres wichti-
ges Signal für die Expression im Mikroorganismus. Dadurch
entstand weiter eine BamHI-Spaltstelle. Diese war für die
10 Klonierung hinter verschiedenen Promotoren (wie trp, tac,T5)
beschriebener Vektoren geeignet.

Die E. coli-Zellen wurden im LB-Vollmedium in Gegenwart
von Ampicillin (50 μg/ml) bis zur mittleren logarithmi-
15 schen Phase angezogen und abzentrifugiert. Das Pellet wurde
in einem Suspensionspuffer mit Guanidiniumhydrochlorid (3 M)
suspendiert (ungefähr 10^{10} Zellen/ml); danach wurde mit
Ultraschall (Sonifier) bis zu einer optischen Dichte (OD_{650})
aufgeschlossen, die etwa 1/3 der optischen Dichte zu Beginn
20 entsprach. Dieser Zellaufschluß wurde abzentrifugiert. Der
Überstand enthielt PTH. Das Protein wurde mit 5-prozentigem
TCA gefällt und in 0,02 n Salzsäure gelöst. TCA-Reste wurden
mit Äther ausgewaschen. Das aus Rohextrakt und nach Extrak-
tion gewonne PTH war immunologisch gegen Antikörper 1-34,
25 28-48 und 48-68 wirksam (RIA) und war im Adenylcyclase-Test
biologisch aktiv.

Das Human-Parathormongen wurde mit einem Vektor verknüpft
(pBR322/SV40-Derivat) und mit Hilfe einer Calciumphosphat-
30 Fällung in Affennierenzellen transformiert. Aus 10^9 Zellen
wurde PTH durch Extraktion gewonnen und im RIA getestet.

Außerdem wurde das Human-Parathormongen (lambda-Humanhybrid-
DNA) mit dem Thymidinkinasegen von Herpes simplex durch
35 Co-Transformation in T3-Zellen transformiert; aus den Tk[+]-Klo-
nen wurde durch DNA-Hybridisieren die Integration des Human-
PTH-Gens identifiziert.

Schema 1
_____

```
Asp Thr Val Lys Val Met Val Val Met Leu Ala Ile Cys Phe
GAC ACA GTT AAA GTA ATG GTT GTC ATG CTT GCA ATT TGT TTT
CTG TGT CAA TTT CAT TAC CAA CAG TAC GAA CGT TAA ACA AAA


Leu Ala Arg Ser Asp Gly Lys Pro Val Lys Lys Arg Ser Val
CTT GCA AGA TCA GAT GGG AAG CCT GTT AAG AAG AGA TCT GTG
GAA CGT TCT AGT CTA CCC TTC GGA CAA TTC TTC TCT AGA CAC


Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Ser
AGT GAA ATA CAG CTT ATG CAT AAC CTG GGC AAA CAC CTG AGC
TCA CTT TAT GTC GAA TAC GTA TTG GAC CCG TTT GTG GAC TCG


Ser Leu Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp
TCT CTG GAG AGA GTG GAA TGG CTG CGA AAG AAG CTG CAG GAT
AGA GAC CTC TCT CAC CTT ACC GAC GCT TTC TTC GAC GTC CTA


Val His Asn Phe Val Val Leu Gly Ala Ser Ile Val His Arg
GTG CAC AAC TTT GTT GTT CTC GGA GCT TCT ATA GTT CAC AGA
CAC GTG TTG AAA CAA CAA GAG CCT CGA AGA TAT CAA GTG TCT


Asp Gly Gly Ser Gln Arg Pro Pro Lys Lys Glu Asp Asn Val
GAT GGT GGT TCC CAG AGA CCC CCA AAA AAG GAA GAC AAT GTC
CTA CCA CCA AGG GTC TCT GGG GGT TTT TTC CTT CTG TTA CAG


Leu Val Glu Ser His Gln Lys Ser Leu Gly Glu Ala Asp Lys
CTA GTT GAG AGC CAT CAA AAA AGT CTC GGA GAA GCA GAT AAA
GAT CAA CTC TCG GTA GTT TTT TCA GAG CCT CTT CGT CTA TTT


Ala Ala Val Gly
GCT GCT GTG GGG
CGA CGA CAC CCC
```

completed

2. Hybridvektor nach Anspruch 1, dadurch g e k e n n -
z e i c h n e t , daß er in E. coli klonierbar ist.

3. In eukaryotischen Zellen klonierbarer und Human-Parathor-
5 mon produzierender Hybridvektor,

(A) dadurch herstellbar, daß man

(a) aus Schweine-Nebenschilddrüsen mRNA isoliert,

(b) die isolierte mRNA als ds-cDNA mit Hilfe eines Vektors
10 in E. coli kloniert,

(c) aus Pools erhaltener Klone Hybridvektor-DNA isoliert,

(d) isolierte Hybridvektor-DNA an einem für jeden Pool eige-
nen Träger fixiert, gemäß (a) isolierte mRNA anlagert und
15 wieder entfernt, entfernte mRNA in Schweine-Parathormon zu
übersetzen versucht, gebildetes Schweine-Parathormon durch
Antikörperfällung nachweist und damit gemäß (b) erhaltene
Klone ermittelt, die Schweine-Parathormongen-Sequenzen auf-
weisen, Hybridvektor-DNA ermittelter und Schweine-Parathormon-
20 gen-Sequenzen aufweisende Klone sequenziert und den oder die
Klone identifiziert, die Hybridvektor-DNA mit Schweine-Parat-
hormon kodierender DNA-Sequenz aufweisen,

(e) Hybridvektor-DNA der gemäß (d) identifizierten Klone
radioaktiv markiert,
25
(f) mit erhaltener radioaktiv markierter Hybridvektor-DNA
eine Human-Genbank screent und

(g) das ermittelte Human-Parathormongen in einen in eukaryo-
tischen Zellen klonierbaren Hybridvektor überführt,

30 (B) und gekennzeichnet durch eine zwischen zwei RI-Schnitt-
stellen liegende DNA-Sequenz, die die folgende DNA-Sequenz
umfaßt, oder einen Unterbereich der zwischen den RI-Schnitt-
stellen liegenden DNA-Sequenz:

35

TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

```
CATTGTAT |
GTAACATA |————————              Intron II ca. 400 bp                   ————
```

```
                  Met Ile Pro Ala Lys Asp Met Ala Lys Val Met
        GTG AAG | ATG ATA CCT GCA AAA GAC ATG GCT AAA GTT ATG
————————    CAC TTC | TAC TAT GGA CGT TTT CTG TAC CGA TTT CAA TAC
```

```
Ile Val Met Leu Ala Ile Cys Phe Leu Thr Lys Ser Asp Gly Lys
ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA AAA TCG GAT GGG AAA
TAA CAG TAC AAC CGT TAA ACA AAA GAA TGT TTT AGC CTA CCC TTT
```

```
Ser Val Lys |
TCT GTT AAG |
AGA CAA TTC |————————————————————————————————————————————————————
```

```
Intron I   | Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn
ca. 30 bp  | AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
———————————| TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG
```

```
Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg
CTG GGA AAA CAT CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT
GAC CCT TTT GTA GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA
```

```
Lys Lys Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro
AAG AAG CTG CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT
TTC TTC GAC GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA
```

```
Leu Ala Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu
CTA GCT CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA
GAT CGA GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT
```

```
Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
CTG TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT
```

```
Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln.
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG TGA
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC ACT
```

```
AAA TGA AAA CAG ATA TTG TCA GAG TTC TGC TCT AGA CAG TGT AGG
TTT ACT TTT GTC TAT AAC AGT CTC AAG ACG AGA TCT GTC ACA TCC
```

```
GCA ACA ATA CAT GCT GCT AAT TCA AAG CTC TAT TAA GAT TTC CAA
CGT TGT TAT GTA CGA CGA TTA AGT TTC GAG ATA ATT CTA AAG GTT
```

```
GTG CCA ATA TTT CTG ATA TAA CAA ACT ACA TGT AAT CCA TCA CTA
CAC GGT TAT AAA GAC TAT ATT GTT TGA TGT ACA TTA GGT AGT GAT
```

```
GCC ATG ATA ACT GCA ATT TTA ATT GAT TAT TCT GAT TCC ACT TTT
CGG TAC TAT TGA CGT TAA AAT TAA CTA ATA AGA CTA AGG TGA AAA
```

```
ATT CAT TTG AGT TAT TTT AAT TAT CTT TTC TAT TGT TTA TTC TTT
TAA GTA AAC TCA ATA AAA TTA ATA GAA AAG ATA ACA AAT AAG AAA
```

```
TTA AAG TAT GTT ATT GCA TAA TTT ATA AAA GAA TAA AAT TCG ACT
AAT TTC ATA CAA TAA CGT ATT AAA TAT TTT CTT ATT TTA AGC TGA
```

```
TTT AAA CCT CTC TTC TAC CTT AAA ATG TAA AAC AAA AAT GTA ATG
AAA TTT GGA GAG AAG ATG GAA TTT TAC ATT TTG TTT TTA CAT TAC
```

```
ATC ATA AGT CTA AAT AAA TGA AGT ATT TCT CAC TCA AA
TAG TAT TCA GAT TTA TTT ACT TCA TAA AGA GTG AGT TT
```

————— Prä-Pro   ———— PTH

4. Hybridvektor nach Anspruch 3, dadurch g e k e n n -
z e i c h n e t , daß er in Humanzellen oder Affenzellen,
wie Affennierenzellen, klonierbar ist.

5. Human-Parathormongen,

(a) herstellbar gemäß Anspruch 3 (A) und

(b) gekennzeichnet durch die zwischen zwei RI-Schnittstellen
liegende DNA-Sequenz gemäß Anspruch 3 (B) oder einen Unter-
bereich davon.

6. Human-Parathormongen nach Anspruch 5, g e k e n n -
z e i c h n e t durch direkte Klonierbarkeit in Humanzellen
oder Affenzellen.

7. Eukaryotische Zellen, insbesondere Tierzellen (wie Affen-
zellen) und Humanzellen, die mit dem Human-Parathormongen
gemäß Anspruch 6 oder 7 Human-Parathormon produzieren.

8. Verwendung der Zellen gemäß Anspruch 7 zum Einsatz bei
parathormondefekten Lebewesen (wie Menschen).

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Patentansprüche

5   1. In prokaryotischen Zellen klonierbarer und Human-Parat-
hormon produzierender Hybridvektor, g e k e n n z e i c h -
n e t   durch folgende Merkmale:

(a) einen Promotor,

10 (b) einen sich an den Promotor anschließenden DNA-Bereich von
0 bis 1000 oder 0 bis 200 Basenpaaren,

(c) eine sich an den DNA-Bereich gemäß (b) anschließende ribo-
somale Bindungsstelle,

(d) einen sich an die ribosomale Bindungsstelle anschließen-
15 den DNA-Bereich von 4 bis 15 Basenpaaren,

(e) ein sich an den DNA-Bereich gemäß (d) anschließendes
Startkodon und

(f) die folgende Human-Parathormon kodierende DNA-Sequenz:

20

```
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His
TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT
AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG GAC CCT TTT GTA

Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu
CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG
GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA TTC TTC GAC

Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro Leu Ala
CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT
GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA GAT CGA

Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu Asp
CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA GAC
GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT CTG

Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT

Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG T
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC A
```

Schema 3:

Klonierungsschema für eine Human-PTH-Genexpression in
E. coli

1) Lambda-Humanhybrid
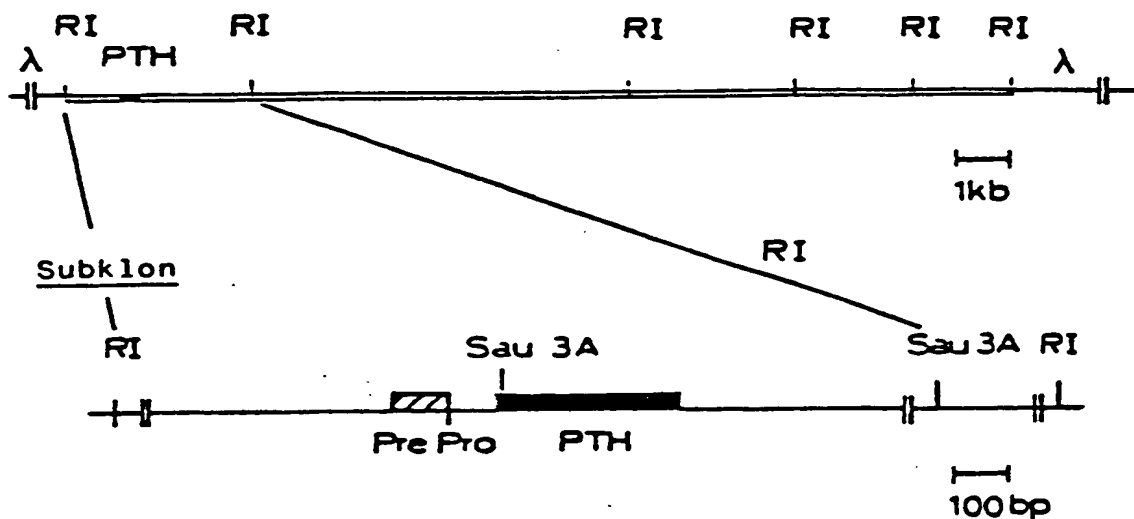
DNA-Sequenz innerhalb dieses mit RI herausgeschnittenen
Bereichs: (folgendes Blatt)

TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

```
CATTGTAT |
GTAACATA |_____    Intron II ca. 400 bp
```

		Met	Ile	Pro	Ala	Lys	Asp	Met	Ala	Lys	Val	Met
GTG	AAG	ATG	ATA	CCT	GCA	AAA	GAC	ATG	GCT	AAA	GTT	ATG
CAC	TTC	TAC	TAT	GGA	CGT	TTT	CTG	TAC	CGA	TTT	CAA	TAC

Ile	Val	Met	Leu	Ala	Ile	Cys	Phe	Leu	Thr	Lys	Ser	Asp	Gly	Lys
ATT	GTC	ATG	TTG	GCA	ATT	TGT	TTT	CTT	ACA	AAA	TCG	GAT	GGG	AAA
TAA	CAG	TAC	AAC	CGT	TAA	ACA	AAA	GAA	TGT	TTT	AGC	CTA	CCC	TTT

```
Ser Val Lys |
TCT GTT AAG |
AGA CAA TTC |_____
```

```
                    | Lys Arg Ser Val Ser Glu Ila Gln Leu Met His Asn
Intron I            | AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
ca. 30 bp _____| TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG
```

Leu	Gly	Lys	His	Leu	Asn	Ser	Met	Glu	Arg	Val	Glu	Trp	Leu	Arg
CTG	GGA	AAA	CAT	CTG	AAC	TCG	ATG	GAG	AGA	GTA	GAA	TGG	CTG	CGT
GAC	CCT	TTT	GTA	GAC	TTG	AGC	TAC	CTC	TCT	CAT	CTT	ACC	GAC	GCA

Lys	Lys	Leu	Gln	Asp	Val	His	Asn	Phe	Val	Ala	Leu	Gly	Ala	Pro
AAG	AAG	CTG	CAG	GAT	GTG	CAC	AAT	TTT	GTT	GCC	CTT	GGA	GCT	CCT
TTC	TTC	GAC	GTC	CTA	CAC	GTG	TTA	AAA	CAA	CGG	GAA	CCT	CGA	GGA

Leu	Ala	Pro	Arg	Asp	Ala	Gly	Ser	Gln	Arg	Pro	Arg	Lys	Lys	Glu
CTA	GCT	CCC	AGA	GAT	GCT	GGT	TCC	CAG	AGG	CCC	CGA	AAA	AAG	GAA
GAT	CGA	GGG	TCT	CTA	CGA	CCA	AGG	GTC	TCC	GGG	GCT	TTT	TTC	CTT

Asp	Asn	Val	Leu	Val	Glu	Ser	His	Glu	Lys	Ser	Leu	Gly	Glu	Ala
GAC	AAT	GTC	TTG	GTT	GAG	AGC	CAT	GAA	AAA	AGT	CTT	GGA	GAG	GCA
CTG	TTA	CAG	AAC	CAA	CTC	TCG	GTA	CTT	TTT	TCA	GAA	CCT	CTC	CGT

Asp	Lys	Ala	Asp	Val	Asn	Val	Leu	Thr	Lys	Ala	Lys	Ser	Gln	
GAC	AAA	GCT	GAT	GTG	AAT	GTA	TTA	ACT	AAA	GCT	AAA	TCC	CAG	TGA
CTG	TTT	CGA	CTA	CAC	TTA	CAT	AAT	TGA	TTT	CGA	TTT	AGG	GTC	ACT

AAA	TGA	AAA	CAG	ATA	TTG	TCA	GAG	TTC	TGC	TCT	AGA	CAG	TGT	AGG
TTT	ACT	TTT	GTC	TAT	AAC	AGT	CTC	AAG	ACG	AGA	TCT	GTC	ACA	TCC
GCA	ACA	ATA	CAT	GCT	GCT	AAT	TCA	AAG	CTC	TAT	TAA	GAT	TTC	CAA
CGT	TGT	TAT	GTA	CGA	CGA	TTA	AGT	TTC	GAG	ATA	ATT	CTA	AAG	GTT
GTG	CCA	ATA	TTT	CTG	ATA	TAA	CAA	ACT	ACA	TGT	AAT	CCA	TCA	CTA
CAC	GGT	TAT	AAA	GAC	TAT	ATT	GTT	TGA	TGT	ACA	TTA	GGT	AGT	GAT
GCC	ATG	ATA	ACT	GCA	ATT	TTA	ATT	GAT	TAT	TCT	GAT	TCC	ACT	TTT
CGG	TAC	TAT	TGA	CGT	TAA	AAT	TAA	CTA	ATA	AGA	CTA	AGG	TGA	AAA
ATT	CAT	TTG	AGT	TAT	TTT	AAT	TAT	CTT	TTC	TAT	TGT	TTA	TTC	TTT
TAA	GTA	AAC	TCA	ATA	AAA	TTA	ATA	GAA	AAG	ATA	ACA	AAT	AAG	AAA
TTA	AAG	TAT	GTT	ATT	GCA	TAA	TTT	ATA	AAA	GAA	TAA	AAT	TCG	ACT
AAT	TTC	ATA	CAA	TAA	CGT	ATT	AAA	TAT	TTT	CTT	ATT	TTA	AGC	TGA
TTT	AAA	CCT	CTC	TTC	TAC	CTT	AAA	ATG	TAA	AAC	AAA	AAT	GTA	ATG
AAA	TTT	GGA	GAG	AAG	ATG	GAA	TTT	TAC	ATT	TTG	TTT	TTA	CAT	TAC

ATC	ATA	AGT	CTA	AAT	AAA	TGA	AGT	ATT	TCT	CAC	TCA	AA
TAG	TAT	TCA	GAT	TTA	TTT	ACT	TCA	TAA	AGA	GTG	AGT	TT

-------- Prä-Pro   ────── PTH

2) erneuter Schnitt (Sau 3A)

| Intron | Lys | | | Arg | Ser | Val |

G AGGAG A AG A
C T CCT CT TCTC T AG

G A T C T G T G ·····
  AC AC ·····

3) Auffüllen mit dG und dA

Ser Val

G A T C T G T G ·····
A G AC AC ·····

4) Abbau mit S1-Nuklease

Ser Val

T C T G T G ·····
AG AC AC ·····

5) Ligation mit DNA-Adaptor

Met Ser Val

CATCGATG T C T G T G ·····
GTAGCTAC AG AC AC ·····

6) erneuter Schnitt (ClaI)

Met Ser Val

C G A T G T C T G T G ·····
T AC AGACAC ·····

7) <u>Subklonieren in die ClaI-Spaltstelle von pBR322 und</u>
   <u>erneuter Schnitt (HindIII)</u>

<div align="right">

ClaI   Met   Ser   Val

AGCTTCATCGATGTCTGTG····
AGTAGCTACAGACAC····

</div>

8) <u>verschiedene Auffüllreaktionen</u>

<div align="center">

Met

TCATCGATG······
AGTAGCTAC······

Met

TTCATCGATG·····
AAGTAGCTAC······

Met

CTTCATCGATG······
GAAGTAGCTAC······

Met

GCTTCATCGATG······
CGAAGTAGCTAC······

Met

AGCTTCATCGATG······
TCGAAGTAGCTAC······

</div>

9) <u>Ligation mit DNA-Adaptoren (2 identischen Linkern)</u>

BamI   Met Ser Val

```
TCCCTAGGGA TCCCTAGGGAGCTTCATCGATGTCTGTG·····
AGGGATCCCT AGGGATCCCTCGAAGTAGCTACAGACAC·····
```
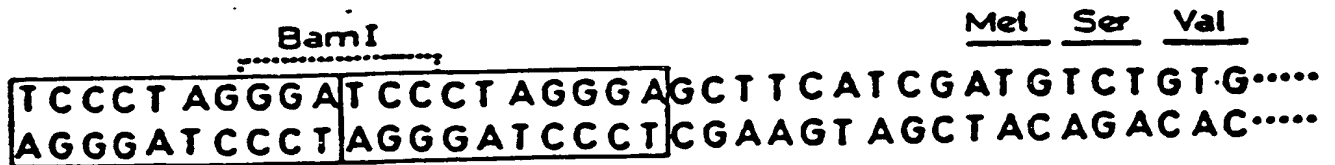
10) <u>erneuter Schnitt (BamI)</u>

Stop   RS   Met Ser Val

```
GATCCCTAG GGAG CTTCATCGATGTCTGTG·····
    GGATC CCTC GAAGTAGCTACAGACAC·····
```

11) <u>Insertion in ein Plasmid nach einem starken Promotor</u>
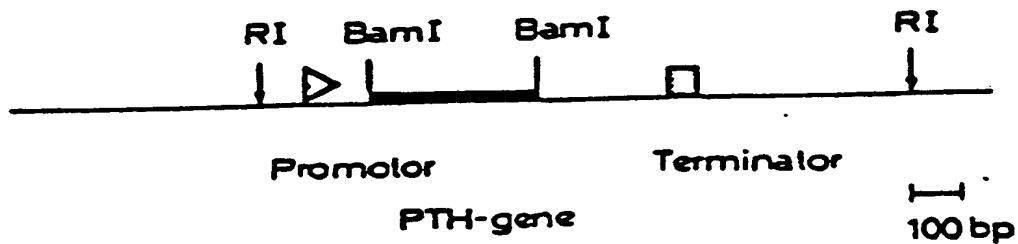
RI   BamI   BamI   RI

Promotor   Terminator

PTH-gene   100 bp

BOETERS, BAUER & PARTNER
PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS

THOMAS-WIMMER-RING 14
D-8000 MÜNCHEN 22

PA: BOETERS, BAUER & PARTNER
THOMAS-WIMMER-RING 14, D-8000 MÜNCHEN 22

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TELEFON: (089) 22 00 92
TELEX: 5 24 878 nm
TELEGRAMME: PROVENTION, MÜNCHEN

— 17 —

Änderungen

1. Seite 1 Zeilen 1 bis 2 sollen lauten: "Human-Parathyroid-hormon (Human-PTH) produzierende Hybridvektoren, Human-Parathyroidhormongen, eukaryotische Zellen mit dem Hybrid-vektor und deren Verwendung.

2. Seite 1 Zeile 4 und fortlaufend   in den Anmeldungsunter-lagen: "Human-Parathyroidhormon" statt "Human-Parathormon".

3. Seite 1 Zeile 5 soll lauten: "Calcium in Knochen. Die Wirkungen und Funktionen von Human-Parathyroidhormon und seinen Agonisten und Antagonisten werden von Dambacher, Praktische Osteologie, Thieme Verlag Stuttgart / New York 1982; Reeve et al, British Medical J. 1340 (1980) 1 - 11; und Potts et al, Advances in Protein Chemistry 35 (1982) 323 - 395 beschrieben.

4. Seite 4 Zeile 4 lautet: "klonieren lassen. Einzelheiten

- 2 -

zum Klonieren und zur Expression von Genen unter der Steue-
rung von E.-coli-Promotoren beispielsweise in Streptomyces
lassen sich bei Chater, Nature 299 (1982) 10 ff finden.
Insbesondere kommt Escherichia coli in Be-".

5. Seite 7 Zeilen 3 bis 4 lauten: "Tierzellen, insbesondere
Wirbeltierzellen, beispielsweise Froschzellen (vgl. Wickens
et al, Nature 285 (1980) 628 bis 634), Säugetierzellen,
Affenzellen, z.B. Affennierenzellen und Humanzellen."

6. Seite 7 Zeile 19 ist der folgende neue Absatz einzu-
setzen: "Schließlich betrifft die Erfindung

- eukaryotische Zellen, wie Tierzellen, insbesondere Wirbel-
  tierzellen, wie Froschzellen, Säugetierzellen, Affenzellen
  und Humanzellen, die Human-Parathyroidhormon mit dem
  Hybridvektor gemäß den Ansprüchen 5, 6, 7 oder 8 produ-
  zieren, und
- die Verwendung der vorstehend angeführten Zellen bei
  Organismen mit mangelhaften Wirkungen und Funktionen
  des Parathyroidhormons, beispielsweise bei Säugetieren,
  insbesondere Menschen.

Bezüglich der Wirkungen und Funktionen eines spezifischen
Parathyroidhormons in Organismen verschiedener Spezies wird
auf Goltzman et al, Pept. Chem. Struct. Biol., Proc. Am.
Pept. Symp. 4th, 1975, p. 571 - 577, 574, verwiesen.

Die im Detail angegebene DNA-Sequenz (Anspruch 1; Anspruch
5 = Schema 2; Schema 1) kann durch DNA-Sequenzen ersetzt
werden, deren Einzelstränge sich mit den Einzelsträngen
der im Detail angegebenen DNA-Sequenz hybridisieren lassen.
Ferner kann jedes Basentriplett durch ein Synonym ersetzt
werden."

7. Seite 5 Zeile 12: "Human-Parathyroidhormongen" anstelle
von "Human-Parathormon".

8. Seiten 6, 11, 13 und 20: "Intron II ca. 4 000 bp" an-
stelle von "Intron II ca. 400 bp"; und "Intron I 103 bp"
anstelle "Intron I ca. 80 bp".

9. Seite 8 drittletzte Zeile: "entgegengesetzten Uhrzeiger-
sinn" statt "Uhrzeigersinn".

10. Seite 9 Zeile 10: "tac, T5" statt "tac T5".

11. Seite 5 Zeilen 12 bis 13 und Seite 19 Zeilen 28 bis 29:
"(g) das ermittelte Human-Parathyroidhormongen (die DNA-
Sequenz zwischen den EcoRI-Schnittstellen gemäß (B)) iso-
liert und in einen Expressionshybridvektor überführt, der
in eukaryotischen Zellen kloniert werden kann,"

12. Seite 5 Zeile 15, Seite 7 Zeile 11, Seite 8 Zeile 14
und Seite 19 Zeile 30: "EcoRI-Schnittstellen" statt "RI-
Schnittstellen".

13. Seite 5 letzte Zeile und Seite 19 Zeilen 32 bis 33:
"EcoRI-Schnittstellen liegenden DNA-Sequenz aufweist, ins-
besondere einen Unterbereich, der Human-Parathyroidhormon
oder dessen Agonisten oder Antagonisten exprimiert:" statt
"RI-Schnittstellen liegenden DNA-Sequenz /aufweist/:".

14. Seite 1 Zeilen 17 bis 21 und Anspruch 1 Zeilen 10 bis
13 sollen lauten: "(b) gegebenenfalls einen sich an den
Promotor anschließenden DNA-Bereich von 1 bis 1000 oder
1 bis 200 Basenpaaren, (c) eine ribosomale Bindungsstelle,
die sich an den DNA-Bereich gemäß (b) oder, sofern (b)
fehlt, an den Promotor gemäß (a) anschließt,".

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15. Seite 4 Zeile 32: "hybridisiert" statt "anlagert".

16. Entfällt.

17. Seite 7 Zeilen 28 bis 29 lauten: "Aus den erhaltenen Hybridklonen wurden die Hybrid-Plasmid-DNAs isoliert und in ihrer Einzelstrangform an einen Träger fixiert. Aus den Nebenschilddrüsen isolierte mRNA wurde mit fixierter Einzelstrang-DNA hybridisiert und entfernt und in".

18. Seite 7 Zeile 34: "und DNA-Sequenzanalyse konnten" statt "konnten".

19. Seite 8 Zeile 2: "ne-cDNA-Parathyroidhormongen" statt "ne-Parathormongen".

20. Seite 8 Zeile 17 soll lauten: "klease Sau3A der Prä-Pro-Teil des PTH-Gens vom Gen"

21. Seite 8 Zeile 16: "(2)" zu streichen.

22. Seite 8 Zeile 18: "getrennt (2)". statt "getrennt. (3)".

23. Seite 8 Zeile 20: "(3) und" statt "und (4)".

24. Seite 8 Zeilen 24, 29 und 33: ". (5)", ". (6 bis 7)" und ". (8)" werden "(4).", "(5)." bzw. "(7).".

25. Seite 8 Zeile 32: "pBR322 (6 bis 7) gegen den Uhrzeigersinn" statt "pBR322 im Uhrzeigersinn".

26. Seite 9 Zeile 4: "(8)." statt ". (9)".

27. Seite 9 Zeile 6: "/TCCCTAGGGA/ (9)." statt "/TCCCTAGGGA/ .".

28. Seite 9 Zeile 9: "BamHI-Spaltstelle (10)" statt "BamHI-Spaltstelle".

29. Seite 9 Zeile 11: "geeignet (11)" statt "geeignet".

30. Seite 8 Zeile 30: "PTH-Genfragment" statt "PTH-Fragment".

31. Seite 9 Zeile 13: "Die transformierten E." statt "Die E.".

32. Seite 9 Zeilen 24 bis 25: "Antikörper (spezifisch zu PTH-Fragmenten AS1-34, AS28-48 und AS48-68) wirksam" statt "Antikörper 1-34, 28-48 und 48-68 wirksam".

33. Seite 9 Zeile 31 soll lauten: "wurde PTH durch Extraktion gewonnen; es war im RIA positiv gegen Antikörper (spezifisch zu den PTH-Fragmenten AS28-48 und AS44-68)."

34. Seite 16: "RI" bedeutet "EcoRI", "BamI" bedeutet "BamHI" und "RS" bedeutet "ribosomale Bindungsstelle".

35. Die folgenden Ansprüche 3 bis 4 sind hinzuzufügen:

"3. Hybridvektor nach Anspruch 1 oder 2, dadurch g e - k e n n z e i c h n e t , daß die spezifische DNA-Sequenz gemäß Anspruch 1 (f) durch eine DNA-Sequenz ersetzt ist, deren Einzelstränge mit den Einzelsträngen der spezifischen DNA-Sequenz hybridisiert werden können, wobei die ersetzende DNA-Sequenz gewünschte Produkte exprimieren kann.

4. Hybridvektor nach Anspruch 1, 2 oder 3, dadurch g e -
k e n n z e i c h n e t , daß ein oder mehrere Basentrip-
letts durch Synonyme ersetzt sind."

35. Der bisherige Anspruch 3 wird Anspruch 5.

36. Auf Seite 21 sollen die bisherigen Ansprüche 4 bis
8 durch die folgenden Ansprüche ersetzt werden:

"6. Hybridvektor nach Anspruch 5, dadurch g e k e n n -
z e i c h n e t , daß er in Tierzellen, insbesondere Wirbel-
tierzellen, Säugetierzellen, Affenzellen, beispielsweise
Affennierenzellen und Humanzellen klonierbar ist.

7. Hybridvektor nach Anspruch 5 oder 6, dadurch g e -
k e n n z e i c h n e t , daß die spezifische DNA-Sequenz
gemäß Anspruch 5 (B) durch eine DNA-Sequenz ersetzt ist,
deren Einzelstränge sich mit den Einzelsträngen der spezi-
fischen DNA-Sequenz hybridisieren lassen, wobei die ersetzen-
de DNA-Sequenz gewünschte Produkte exprimieren kann.

8. Hybridvektor nach Anspruch 5, 6 oder 7, dadurch g e -
k e n n z e i c h n e t , daß ein oder mehrere Basentrip-
letts durch Synonyme ersetzt sind.

9. Human-Parathyroidhormongen:

(a) herstellbar gemäß Anspruch 5 (A) und
(b) gekennzeichnet durch die zwischen zwei EcoRI-Schnitt-
stellen liegende DNA-Sequenz gemäß Anspruch 5 (B) oder
einen Unterbereich davon.

- 7 -

10. Eukaryotische Zellen, insbesondere Tierzellen, beispielsweise Wirbeltierzellen, Säugetierzellen, Affenzellen und Humanzellen, die Human-Parathyroidhormon mit dem Hybridvektor gemäß Anspruch 5, 6, 7 oder 8 produzieren.

11. Verwendung der Zellen gemäß Anspruch 10 für Organismen (beispielsweise Säugetiere, insbesondere Menschen) mit Parathyroid-Mangelwirkungen oder Parathyroid-Mangelfunktionen."

TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

```
CATTGTAT |
GTAACATA |                          Intron II ca.  400 bp
```

			Met	Ile	Pro	Ala	Lys	Asp	Met	Ala	Lys	Val	Met
	GTG	AAG	ATG	ATA	CCT	GCA	AAA	GAC	ATG	GCT	AAA	GTT	ATG
		CAC	TTC	TAC	TAT	GGA	CGT	TTT	CTG	TAC	CGA	TTT	CAA	TAC

Ile	Val	Met	Leu	Ala	Ile	Cys	Phe	Leu	Thr	Lys	Ser	Asp	Gly	Lys
ATT	GTC	ATG	TTG	GCA	ATT	TGT	TTT	CTT	ACA	AAA	TCG	GAT	GGG	AAA
TAA	CAG	TAC	AAC	CGT	TAA	ACA	AAA	GAA	TGT	TTT	AGC	CTA	CCC	TTT

```
Ser Val Lys |
TCT GTT AAG |
AGA CAA TTC |
```

```
Intron I     | Lys Arg Ser Val Ser Glu Ila Gln Leu Met His Asn
ca. 30 bp    | AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
             | TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG
```

Leu	Gly	Lys	His	Leu	Asn	Ser	Met	Glu	Arg	Val	Glu	Trp	Leu	Arg
CTG	GGA	AAA	CAT	CTG	AAC	TCG	ATG	GAG	AGA	GTA	GAA	TGG	CTG	CGT
GAC	CCT	TTT	GTA	GAC	TTG	AGC	TAC	CTC	TCT	CAT	CTT	ACC	GAC	GCA

Lys	Lys	Leu	Gln	Asp	Val	His	Asn	Phe	Val	Ala	Leu	Gly	Ala	Pro
AAG	AAG	CTG	CAG	GAT	GTG	CAC	AAT	TTT	GTT	GCC	CTT	GGA	GCT	CCT
TTC	TTC	GAC	GTC	CTA	CAC	GTG	TTA	AAA	CAA	CGG	GAA	CCT	CGA	GGA

Leu	Ala	Pro	Arg	Asp	Ala	Gly	Ser	Gln	Arg	Pro	Arg	Lys	Lys	Glu
CTA	GCT	CCC	AGA	GAT	GCT	GGT	TCC	CAG	AGG	CCC	CGA	AAA	AAG	GAA
GAT	CGA	GGG	TCT	CTA	CGA	CCA	AGG	GTC	TCC	GGG	GCT	TTT	TTC	CTT

Asp	Asn	Val	Leu	Val	Glu	Ser	His	Glu	Lys	Ser	Leu	Gly	Glu	Ala
GAC	AAT	GTC	TTG	GTT	GAG	AGC	CAT	GAA	AAA	AGT	CTT	GGA	GAG	GCA
CTG	TTA	CAG	AAC	CAA	CTC	TCG	GTA	CTT	TTT	TCA	GAA	CCT	CTC	CGT

Asp	Lys	Ala	Asp	Val	Asn	Val	Leu	Thr	Lys	Ala	Lys	Ser	Gln	
GAC	AAA	GCT	GAT	GTG	AAT	GTA	TTA	ACT	AAA	GCT	AAA	TCC	CAG	TGA
CTG	TTT	CGA	CTA	CAC	TTA	CAT	AAT	TGA	TTT	CGA	TTT	AGG	GTC	ACT

AAA	TGA	AAA	CAG	ATA	TTG	TCA	GAG	TTC	TGC	TCT	AGA	CAG	TGT	AGG
TTT	ACT	TTT	GTC	TAT	AAC	AGT	CTC	AAG	ACG	AGA	TCT	GTC	ACA	TCC

GCA	ACA	ATA	CAT	GCT	GCT	AAT	TCA	AAG	CTC	TAT	TAA	GAT	TTC	CAA
CGT	TGT	TAT	GTA	CGA	CGA	TTA	AGT	TTC	GAG	ATA	ATT	CTA	AAG	GTT

GTG	CCA	ATA	TTT	CTG	ATA	TAA	CAA	ACT	ACA	TGT	AAT	CCA	TCA	CTA
CAC	GGT	TAT	AAA	GAC	TAT	ATT	GTT	TGA	TGT	ACA	TTA	GGT	AGT	GAT

GCC	ATG	ATA	ACT	GCA	ATT	TTA	ATT	GAT	TAT	TCT	GAT	TCC	ACT	TTT
CGG	TAC	TAT	TGA	CGT	TAA	AAT	TAA	CTA	ATA	AGA	CTA	AGG	TGA	AAA

ATT	CAT	TTG	AGT	TAT	TTT	AAT	TAT	CTT	TTC	TAT	TGT	TTA	TTC	TTT
TAA	GTA	AAC	TCA	ATA	AAA	TTA	ATA	GAA	AAG	ATA	ACA	AAT	AAG	AAA

TTA	AAG	TAT	GTT	ATT	GCA	TAA	TTT	ATA	AAA	GAA	TAA	AAT	TCG	ACT
AAT	TTC	ATA	CAA	TAA	CGT	ATT	AAA	TAT	TTT	CTT	ATT	TTA	AGC	TGA

TTT	AAA	CCT	CTC	TTC	TAC	CTT	AAA	ATG	TAA	AAC	AAA	AAT	GTA	ATG
AAA	TTT	GGA	GAG	AAG	ATG	GAA	TTT	TAC	ATT	TTG	TTT	TTA	CAT	TAC

ATC	ATA	AGT	CTA	AAT	AAA	TGA	AGT	ATT	TCT	CAC	TCA	AA
TAG	TAT	TCA	GAT	TTA	TTT	ACT	TCA	TAA	AGA	GTG	AGT	TT

----- Prä-Pro   ——— PTH

EVALUATION OF US PATENT NO 4,698,321;   DATAPATENT OCT. 1987.

The inventors are Robert M. Neer, John T. Potts Jr., and David M. Slovic. They claim to have sufficient originality in developing a method which is aimed to increase bone mass in humans afflicted with osteoporosis or similar disease. The method comprises of administering to humans a combination treatment comprising of parathyroid hormone or physiologically active fragment thereof in combination with either a) a hydroxylated vitamin D compound (of that they mention 1α, 1,25 dihydroxy- and 25 hydroxy-vitamin D_3 and vitamin D_2) or related functional analogs or b) with or without a dietary calcium supplement.

They give 31 different claims which extend the above general claim in direction where they deal with all kinds of formulation procedures, concentration variations as well as different supplement of salt and vitamins.

Conclusion.

It is important to realize that in their study from which they show one figure relating to osteoporotic men only, they have only used the biologically active 1-34 amino acid synthetic fragment of hPTH. They have carried out an intermittent treatment where PTH fragment has been given by injections on different days while Vit. D has been given as tablets in alternating scheme with the peptide. They had to use the Vit. D substitution because otherwise the patient would not aquire positive calcium balance, meaning that the increase in trabecular bone e.g. in the spine, would otherwise be the result of redistribution of calcium within the body.

The nature has used millions of years of creating the best PTH and obviously the final result emerge as a 1-84 amino acid peptide and not as shorter fragments. Since the availability of the intact hormone is scarce and the peptide thus is expensive, a full study of its potential in this disease as well as in others, in comparison to the shorter peptide, has not been carried out. At present it is well known that the half-life in circulation of the 1-34 amino acid fragments is much reduced compared to the intact hormone. It is a puzzle that the 1-34 fragment does not increase the endogenous production of the active Vit. D3 in the kidney which is one of the hormone's _major_ physiological actions. If, however, future studies using the intact hormone show that humans are in fact developing a positive calcium balance on treatment with this hormone alone, then we would have a complete physiological situation and the need for Vit. D supplement other than that present in normal food would be eliminated. Also, because of its well known longer survival time in the circula-tion, the formulation in general would be more apt to ease the work of drug administration.

(12) UK Patent Application (19) GB (11) 2 092 596 A

(54) **Process for the production of human parathyroid hormone**

(57) A process for the production of human parathyroid hormone (hPTH), comprising *in vivo* multiplication of human lymphoblastoid cells capable of producing said hormone, using a non-human warm-blooded animal, and *in vitro* cultivation of the multiplied human lymphoblastoid cells to produce hPTH.

GB 2 092 596 A

SPECIFICATION
Process for the production of human
parathyroid hormone

The present Invention relates to a process for
5 the production of human parathyroid hormone
(abbreviated as hPTH hereinafter).

hPTH is a hormone, secreted by the parathyroid
glands, that regulates the synthesis of activated
vitamin D_3, stimulates the release of calcium from
10 bones and leads to an increase in the calcium level
of the blood. No process for the mass production
of low-cost hPTH has been established to date.

We have investigated processes for the mass
production of hPTH and have unexpectedly found
15 that certain human lymphoblastoid cells capable
of producing hPTH are suitable for the mass
production of hPTH owing to their very high
multiplication efficiency and high rate of hPTH
production per cell.

20   According to the present invention there is
provided a process for the production of hPTH,
which process comprises multiplying human
lymphoblastoid cells capable of producing said
hormone by transplanting said cells to a non-
25 human warm-blooded animal body, or
alternatively by allowing said cells to multiply
within a device in which the nutrient body fluid of
a non-human warm-blooded animal is supplied to
said cells, and allowing the human lymphoblastoid
30 cells multiplied by either of the above
multiplication procedures to release said hormone.

The process according to the invention provides
an extremely high hPTH yield, requires much less
nutrient medium containing expensive serum for
35 the cell multiplication or no such medium, and
renders much easier the maintenance of the
culture medium during the cell multiplication than
in in vitro tissue culture. Particularly, any human
lymphoblastoid cells capable of producing hPTH
40 can be multiplied easily while utilising the nutrient
body fluid supplied from the non-human warm-
blooded animal body by transplanting said cells to
the animal body, or suspending the cells in a
conventional diffusion chamber devised to receive
45 the nutrient body fluid of the animal, and feeding
the animal In the usual way. Also, in the present
process one obtains a more stable and a higher
rate of cell multiplication, and a higher hPTH
production per cell.

50   As regards the human lymphoblastoid cells
which may be used in the present invention, any
human lymphoblastoid cells can be used as long
as they produce hPTH and multiply rapidly in the
non-human warm-blooded animal body.

55 Preferable human lymphoblastoid cells are those
introduced with hPTH production governing
genetic sites of human cells which inherently
produce hPTH such as normal or transformed
parathyroid cells, or human cells which produce
60 ectopic hPTH such as lung carcinoma cells,
ovarian tumor cells, kidney carcinoma cells or liver
carcinoma cells by means of cell fusion using
polyethylene glycol, or by genetic recombination
techniques using DNA ligase, nuclease and DNA

65 polymerase; and other human lymphoblastoid
cells which produce ectopic hPTH. Since the use
of such human lymphoblastoid cells results in the
formation of easily disaggregatable massive
tumors when the cells are transplanted to the
70 animal body, and the massive tumors are barely
contaminated with the host animal cells, the
multiplied live human lymphoblastoid cells can be
harvested easily.

Any non-human warm-blooded animal can be
75 used to perform the process of the present
invention as long as the human lymphoblastoid
cells multiply therein. Examples of suitable
animals are poultry such as chickens or pigeons,
and mammals such as dogs, cats, monkeys, goats,
80 pigs, cows, horses, guinea pigs, rats, hamsters,
mice and nude mice. Since transplantation of the
human lymphoblastoid cells gives rise to
undesirable immunoreactions, the use of a
newborn or infant animal, or of an animal in the
85 youngest possible stage, for example, in the form
of an egg, embryo or foetus, is desirable. In order
to reduce the incidence of immunoreactions as
much as possible, prior to the cell transplantation
the animal may be treated with Xray of γ-ray
90 irradiation, at about 200—600 rem, or with an
injection of antiserum or an immunosuppressive
agent prepared according to conventional
methods. Nude mice exhibit weak
immunoreactions; consequently, any human
95 lymphoblastoid lines capable of producing hPTH
can advantageously be transplanted into, and
rapidly multiplied in, nude mice without subjecting
the mice to a pretreatment for suppressing
immunoreactions.

100   Stabilised cell multiplication and enhancement
of hPth production can be both carried out by
repeated transplantation using combination(s) of
different non-human warm-blooded animals; the
objectives are attainable first by implanting said
105 cells in hamsters and multiplying the cells therein,
then by reimplanting the cells in nude mice.
Repeated transplantation may be carried out with
animals of the same class or division as well as
those of the same species or genus.

110   The human cells to be multiplied can be
implanted in any site of the animal as long as they
multiply at that site; for example, in the allantoic
cavity, or intraveneously, intraperitoneally, or
subcutaneously.

115   In addition to the above-mentioned direct cell
transplantation, any conventional human
lymphoblastoid lines capable of producing hPTH
can be multiplied easily by using the nutrient body
fluid supplied from the animal body by embedding,
120 for example, intraperitoneally, in the animal body a
conventional diffusion chamber, of various shapes
and sizes, and equipped with a porous membrane
filter, ultra filter or hollow fiber having a pore size
of about 10^{-7} to 10^{-5} m in diameter which
125 prevents ingress of the host animal cells into the
chamber but permits the cells to be supplied with
the nutrient body fluid of the animal. Furthermore,
the diffusion chamber can be designed, if desired,
so as to enable observation of the cell suspension

in the chamber through transparent side
window(s) provided on the chamber wall(s), and
so as to enable replacement and exchange with a
fresh chamber. In this way cell production per host
5 can be increased to even higher levels over the
period of the animal's life without any sacrifice of
the host animal. When such a diffusion chamber is
used, since little immunoreaction arises owing to
the absence of direct contact of the human cells
10 with the host animal cells, the multiplied human
lymphoblastoid cells can be harvested easily, and
any non-human warm-blooded animal can be
used as the host in the present process without
the need for any pretreatment to reduce
15 immunoreactions.
Feeding of the host animal can be carried out
by conventional methods even after cell
transplantation, and no special care is required.
Maximum cell multiplication can be attained
20 within 1—20 weeks, generally 1—5 weeks, after
the cell transplantation.
According to the invention, the number of the
human lymphoblastoid cells obtained per host
ranges from about 10^7 to 10^{12} or more. In other
25 words, the number of the human lymphoblastoid
cells implanted in the animal body increases about
10^2 to 10^7 times or more, or about 10^1 to 10^6
times or more than that attained by *in vitro* tissue
culture method using a nutrient medium; the
30 human lymphoblastoid cells can therefore be used
advantageously in the production of hPTH.
As regards the method by which the human
lymphoblastoid cells multiplied by either of the
above described procedures are allowed to release
35 hPTH, any methods can be employed as long as
the said human cells release the desired hormone
thereby. For example, human lymphoblastoid cells,
obtained by multiplying in ascite in suspension
and harvesting from said ascite, or by extracting
40 the massive tumor formed subcutaneously and
harvesting after the disaggregation of the massive
tumor, are suspended to give a cell concentration
of about 10^4 to 10^8 cells per ml in a nutrient
medium, prewarmed at a temperature of about
45 20—40°C, and then incubated at this
temperature for about 1 to 100 hours to produce
hPTH. During the incubation, enhancement of
hPTH production may be carried out by including
one or more of an amino acid such as glycine,
50 leucine, lysine, arginine and cysteine; an inorganic
salt such as sodium chloride, potassium chloride,
calcium chloride and magnesium sulfate; and a
hormone such as dopamine, isoproterenol,
epinephrine and norepinephrine.
55 The hPTH thus obtained can be collected easily
by purification and separation techniques using
conventional procedures such as salting-out,
dialysis, filtration, centrifugation, concentration
and lyophilisation. If a more highly purified hPTH
60 preparation is desirable, a preparation of the
highest purity can be obtained by the above-
mentioned techniques in combination with other
conventional procedures such as adsorption and
desorption with ion exchange, gel filtration, affinity
65 chromatography, isoelectric point fractionation

and electrophoresis.
The hPTH preparation thus obtained can be
used advantageously alone or in combination with
one or more agents for injection, or for external,
70 internal or diagnostical administration in the
prevention and treatment of human diseases.
The following Examples illustrate the present
invention.
In this specification, hPTH production was
75 determined by a bioassay method as described in
J. A. Parsons *et al.*, Endocrinology, Vol 92, pp.
454—462 (1973), and is expressed by weight in
terms of the standard hPTH preparation, assigned
1,300 USP units per mg, available from the
80 National Institute of Health (USA).

EXAMPLE 1
Disaggregated human parathyroid tumor
cells — extracted from a patient suffering from
parathyroid tumor and minced — and a human
85 leukemic lymphoblastoid line Namalwa were
suspended together in a vessel with a salt
solution, containing 140 mM NaCl, 54 mM KCl,
1 mM NaH_2PO_4 and 2 mM $CaCl_2$, to give a
respective cell concentration of about 10^4 cells per
90 ml. The ice-chilled cell suspension was mixed with
a fresh preparation of the same salt solution
containing UV-irradiation preinactivated Sendai
virus, transferred into a 37°C incubator five
minutes after the mixing, and stirred therein for 30
95 minutes to effect cell fusion, thereby introducing
the ability of the human parathyroid tumor cells of
producing hPTH into the human leukemic
lymphoblastoid line.
After cloning according to conventional
100 methods the hybridoma cell strain capable of
producing hPTH, the hybridoma cells strain was
implanted intraperitoneally in adult nude mice
which were then fed in the usual way for five
weeks. The resulting massive tumors, about 15 g
105 each, were extracted and disaggregated by
mincing and trypsinizing.
After washing the cells with Earle's 199
medium (pH 7.2), supplemented with 10 v/v %
foetal calf serum, the cells were resuspended to
110 give a cell concentration of about 10^5 cells per ml
in a fresh preparation of the same medium which
contained 30 mM L-arginine and 20 mM $CaCl_2$,
and then incubated at 37°C for 40 hours to
produce hPTH. Thereafter, the cells were treated
115 ultrasonically, and the hPTH in the resulting
supernatant was determined. The hPTH
production was about 830 ng per ml cell
suspension.
Control cells were obtained by cultivating *in*
120 *vitro* the human parathyroid cells at 37°C in
Earle's 199 medium (pH 7.2), supplemented with
10 v/v % foetal calf serum. These cells were
treated similarly as described above to produce
hPTH. The hPTH production was only about 4 ng
125 per ml cell suspension.

EXAMPLE 2
Disaggregated human kidney carcinoma
cells — extracted from a patient suffering from

kidney carcinoma and minced — and a human leukemic lymphoblastoid line JBL were fused in a manner similar to that described in Example 1, thereby introducing the ability of the human

5 kidney carcinoma cells of producing hPTH into the human leukemic lymphoblastoid line.

After cloning according to conventional methods the hybridoma cell strain capable of producing hPTH, the hybridoma cell strain was

10 implanted subcutaneously in newborn hamsters which had been preinjected with an antiserum (prepared from rabbits using conventional methods) so as to reduce the immunoreactions of the rabbits. The rabbits were then fed in the usual

15 way for three weeks.

The resulting massive tumors, formed subcutaneously and about 10 g each, were extracted and disaggregated by mincing and suspending in a physiological saline solution

20 containing collagenase.

After washing the cells with Eagle's minimal essential medium (pH 7.4), supplemented with 5 v/v % human serum, the cells were resuspended to give a cell concentration of about 10^6 cells per

25 ml in a fresh preparation of the same medium which contained 20 mM $CaCl_2$ and 20 mM dopamine, and then incubated at 37°C for 20 hours to produce hPTH. The hPTH production was about 1.3 μg per ml cell suspension.

30   Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line JLB. The control cells were treated similarly as described above. The hPTH production was only

35 about 16 ng per ml cell suspension.

EXAMPLE 3

Newborn rats were implanted intravenously with a human leukemic lymphoblastoid line BALL-1 into which the ability of human ovarian tumor

40 cells of producing hPTH had been introduced in a manner similar to that described in Example 1, and then fed in the usual way for four weeks.

The resulting massive tumors, about 30 g each, were extracted and treated similarly as described

45 in Example 1 to produce hPTH. The hPTH production was about 900 ng per ml suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line

50 BALL-1. These control cells were treated similarly as described above. The hPTH production was only about 10 ng per ml cell suspension.

EXAMPLE 4

After about 400 rem X-ray irradiation of adult

55 mice to reduce their immunoreactions, the mice were implanted subcutaneously with a human leukemic lymphoblastoid line NALL-1 into which the ability of human lung carcinoma cells to produce hPTH had been introduced in a manner

60 similar to that described in Example 1. The mice were then fed in the usual way for three weeks.

The resulting massive tumors, formed subcutaneously and about 15 g each, were

extracted and treated similarly as described in

65 Example 2 to produce hPTH. The hPTH production was about 1.2 μg per ml cell suspension.

Control cells were obtained similarly as described in Example 1 by cultivating *in vitro* the fused human leukemic lymphoblastoid line

70 NALL-1. The control cells were treated similarly as described above. The hPTH production was only about 20 ng per ml cell suspension.

EXAMPLE 5

A human leukemic lymphoblastoid line TALL-1

75 into which the ability of the human parathyroid tumor cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was suspended in physiological saline solution, and the resulting cell suspension was transferred

80 into a plastic cylindrical diffusion chamber (inner volume: about 10 ml) and equipped with a membrane filter having a pore size of about 0.5 μ in diameter. After intraperitoneal embedding of the chamber into an adult rat, the rat was fed in

85 the usual way for four weeks, and the chamber was removed.

The human lymphoblastoid cell density in the chamber attained by the above operation was about 6×10^8 cells per ml which was about 10^2

90 times higher or more than that attained by *in vitro* cultivation using a CO_2 incubator. The human lymphoblastoid cells thus obtained were treated similarly as described in Example 2 to produce hPTH. The hPTH production was about 1.1 μg per

95 ml cell suspension.

Control cells were obtained by suspending the human parathyroid tumor cells in physiological saline solution, transferring the resulting cell suspension in the chamber, embedding

100 intraperitoneally the chamber into an adult rat, feeding the rat in the usual way for four weeks, and harvesting the multiplied human lymphoblastoid cells (human cell density, about 8×10^8 cells per ml). The control cells were

105 treated similarly as described above. The hPTH production was only about 3 μg per ml cell suspension.

EXAMPLE 6

A human leukemic lymphoblastoid line JBL into

110 which the ability of the human lung carcinoma cells to produce hPTH had been introduced in a manner similar to that described in Example 1 was implanted in the allantoic cavities of embryonated eggs which had been preincubated at 37°C for

115 five days. After further incubation of the eggs at this temperature for an additional one week, the chamber was removed.

The multiplied human lymphoblastoid cells thus obtained were treated similarly as described in

120 Example 1 to produce hPTH. The hPTH production was about 700 ng per ml cell suspension.

In a control experiment in which the human lung carcinoma cells were implanted in the allantoic cavities of embryonated eggs, no cell

125 multiplication was observed.

CLAIMS

1. A process for the production of human parathyroid hormone (hPTH), which process comprises multiplying human lymphoblastoid cells
5 capable of producing said hormone by transplanting said cells to a non-human warm-blooded animal body, and allowing the multiplied human lymphoblastoid cells to release said hormone; or multiplying human lymphoblastoid
10 cells capable of producing said hormone by allowing said cells to multiply within a device in which the nutrient body fluid of a non-human warm-blooded animal is supplied to said cells, and allowing the multiplied human lymphoblastoid
15 cells to release said hormone.

2. A process according to Claim 1, wherein the human lymphoblastoid cells are hybridoma cells obtained by cell fusion of a human lymphoblastoid line with human cells capable of producing hPTH.
20 3. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human parathyroid tumor cells.

4. A process according to Claim 2, wherein the
25 hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human kidney tumor cells.

5. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion
30 of a human lymphoblastoid line with human ovarian tumor cells.

6. A process according to Claim 2, wherein the hybridoma cells are those obtained by cell fusion of a human lymphoblastoid line with human lung
35 carcinoma cells.

7. A process according to any one of Claims 2 to 6 wherein the human lymphoblastoid line is a human leukemic lymphoblastoid line.

8. A process according to any one of Claims 2
40 to 7, wherein the human lymphoblastoid line is Namalwa, BALL-1, NALL-1, TALL-1 or JBL.

9. A process according to any one of the preceding Claims, wherein the multiplied human lymphoblastoid cells are allowed to release hPTH
45 in the presence of one or more of glycine, leucine, lysine, arginine, cysteine; sodium chloride, potassium chloride, calcium chloride, magnesium sulfate; dopamine, isoproterenol, epinephrine and norepinephrine.
50 10. A process according to any one of the preceding claims, wherein the non-human warm-blooded animal is a chicken, pigeon, dog, cat, monkey, goat, pig, cow, horse, guinea pig, rat, hamster, mouse or nude mouse.
55 11. A process according to Claim 1 substantially as hereinbefore described in any one of the Examples.

12. Human parathyroid hormone whenever prepared by a process as claimed in any one of the
60 preceding claims.

Characterization of a Novel Parathyroid Hormone (PTH) Receptor with Specificity for the Carboxyl-Terminal Region of PTH-(1–84)*

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ABSTRACT

Carboxyl-terminal fragments of PTH (C-PTH) appear to have biological properties different from those mediated by the amino-terminal portions of PTH and PTH-related peptide (PTHrP). To characterize a C-PTH receptor that may be involved in mediating these functions, we performed RRAs and affinity cross-linking studies with several clonal cell lines. Radiolabeled recombinant [Leu⁸,¹⁸,Tyr³⁴]human PTH-(1–84) [mutPTH-(1–84)] and [Tyr³⁴]human PTH-(19–84) [mutPTH-(19–84)] showed little or no specific binding to stably expressed recombinant PTH/PTHrP receptors. However, high affinity binding was observed using osteoblast-like osteosarcoma (ROS 17/2.8) and rat parathyroid (PT-r3) cells. The apparent K_d values were 20–30 nM for PTH-(1–84), mutPTH-(1–84), and mutPTH-(19–84), respectively; 400–800 nM for PTH-(39–84); and more than 5000 nM for PTH-(53–84). [Nle⁸,¹⁸,Tyr³⁴]bovine PTH-(1–34)amide [PTH-(1–34)], PTH-(44–68), PTHrP-(37–74), and PTHrP-(109–141) showed no displacement of either radioligand. C-PTH receptor number was increased up to 2-fold by pretreating ROS 17/2.8 cells with increasing doses of PTH-(1–34), PTH-(1–84), or 8-bromo-cAMP, whereas no change was observed in response to dexamethasone or PTH-(39–84). Cross-linking studies using radiolabeled mutPTH-(1–84) or mutPTH-(19–84) revealed specific labeling of two proteins in ROS 17/2.8 cells that were approximately 40 and 90 kilodaltons in size (including the radioligand of approximately 10 kilodaltons). The intensity of affinity labeling of both proteins was dose dependently inhibited by increasing concentrations of unlabeled PTH-(1–84) and several carboxyl-terminal PTH-(1–84) fragments, but not by PTH-(1–34). Similar studies with PT-r3 cells revealed only a single protein band of about 90 kilodaltons. These data indicate that the carboxyl-terminal portion of PTH-(1–84) binds specifically to a unique receptor/binding protein distinct from the previously isolated PTH/PTHrP receptor. (Endocrinology 136: 4732–4740, 1995)

PTH is the major regulator of calcium and phosphate homeostasis through its actions on bone and kidney. Intact PTH comprises 84 amino acids, but only the amino-terminal 1–34 fragment is necessary and sufficient for evoking all classical functions of PTH (1). This amino-terminal portion of the PTH molecule shares significant structural and functional homology with PTH-related peptide (PTHrP), which is the major cause of the syndrome of humoral hypercalcemia of malignancy (2). Due to this similarity, amino-terminal analogs of both ligands bind with similar or identical affinity to the common PTH/PTHrP receptor that is a member of a novel family of G protein-coupled receptors (3).

PTH-(1–84) is the predominant secretory product of parathyroid glands. However, fragmentation of the intact peptide occurs in several peripheral organs, most notably liver and kidney (1), and at least its hepatic metabolism appears to be influenced by extracellular calcium concentrations (4). Carboxyl-terminal fragments are predominantly eliminated by the kidney, which explains their significant accumulation in patients with renal failure (5). The increased secretion of intact PTH and/or these poorly defined carboxyl-terminal PTH fragments may be at least partially responsible for some of the uremia-related metabolic disturbances (6, 7).

Large portions of the carboxyl-terminus of PTH are well conserved in several mammalian species and in chicken, which suggests that this part of the PTH molecule serves important biological functions (8). This assumption is supported by earlier studies that showed specific binding sites for carboxyl-terminal PTH fragments in renal plasma membranes and osteoblast-like cells (9, 10). Furthermore, a synthetic fragment of PTH, PTH-(53–84), increases alkaline phosphatase activity in dexamethasone-treated ROS 17/2.8 cells (10, 11), and more recent studies showed that PTH-(39–84) and PTH-(53–84), but not shorter or unrelated peptides, dose-dependently stimulate the differentiation of osteoclast precursors into osteoclast-like cells (12). These data suggest that specific receptors for the carboxyl-terminal portion of PTH-(1–84) (C-PTH receptor) not only exist on osteoblasts, but are also present on either osteoclast precursors or mature osteoclasts, as indicated by previous immunohistochemical studies (13).

To characterize such a receptor with specificity for the carboxyl-terminal portion of PTH that could be implicated in some of the above observations, we generated recombinant analogs of human PTH-(1–84) that interact poorly or not at all with the receptor that recognizes amino-terminal PTH and PTHrP. RRAs with ROS 17/2.8 and PT-r3 cells showed high affinity binding of radioiodinated PTH-(1–84) analogs that was inhibited by PTH-(1–84) and several carboxyl-terminal PTH-(1–84) fragments, but not by amino-terminal or midregional PTH analogs. Furthermore, affinity cross-linking studies with ¹²⁵I-labeled recombinant [Leu⁸,¹⁸, Tyr³⁴]human (h) PTH-(1–84) [mutPTH-(1–84)] and

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FIG. 1. Schematic representation of the plasmids encoding the fusion proteins hGH/[Met^{8,18},Phe^{34}]hPTH-(1–84) (pGTP-C2), hGH/[Met^{8,18},Tyr^{34}]hPTH-(1–84) (pGTP-Tyr^{34}), and hGH/[Leu^{8,18},Tyr^{34}]hPTH-(1–84) (pGTP-Leu^{8,18},Tyr^{34}). Amino acids are shown in single letter code. The (Gly-Gly-Thr) spacer that introduces a *KpnI* restriction site and the thrombin recognition sequence (Leu-Val-Pro-Arg) are indicated; additional unique restriction sites that do not change the encoded amino acid sequence are shown.
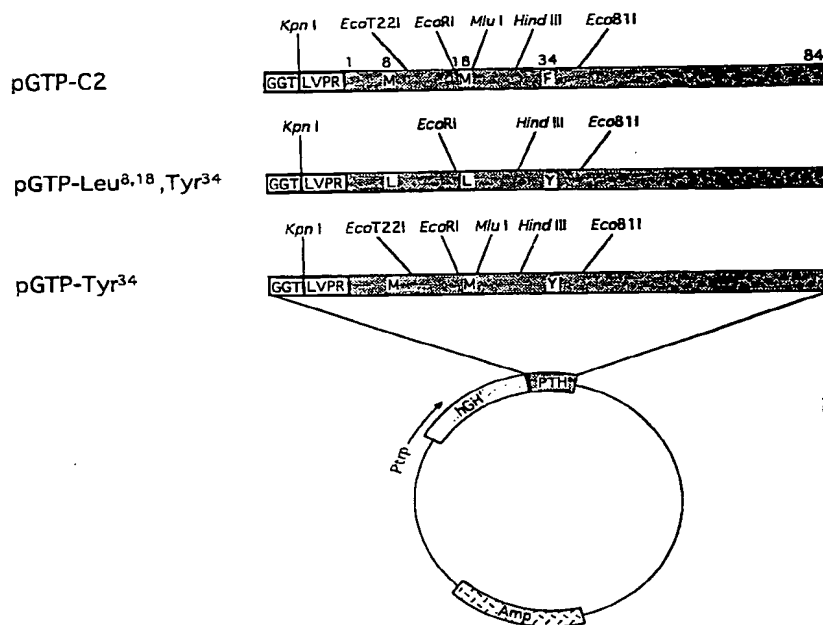
[Tyr^{34}]hPTH-(19–84) [mutPTH-(19–84)] revealed evidence for an approximately 90-kilodalton (kDa) C-PTH receptor protein in both cell lines. In ROS 17/2.8 cells, we identified an additional radiolabeled protein that is about 40 kDa in size.

vine parathyroid glands and purified to homogeneity as described previously (15). The human PTHrP fragments, PTHrP-(37–74) and PTHrP-(109–141), were kindly provided by Dr. A. F. Stewart (Yale University. New Haven, CT). The synthesis of [N^{α}-(4-azido-2-nitrophenyl)Ala^{1},Tyr^{36}]PTHrP-(1–36)amide (compound D-2) was previously described (16).

Materials and Methods

Synthetic peptides and highly purified bovine (b) PTH-(1–84)

Most synthetic and recombinant peptides that were used in this study are listed in Table 1 together with their abbreviated names. [Nle^{8,18},Tyr^{34}]bPTH-(1–34)amide, [Asn^{76}]hPTH-(53–84), and hPTH-(44–68) were purchased from Bachem (Torrance, CA), [Asn^{76}]hPTH-(39–84) was obtained from Peninsula Laboratories (Belmont, CA). Dr. H.T. Keutmann (Endocrine Unit, Massachusetts General Hospital, Boston, MA) provided [Tyr^{36}]hPTHrP-(1–36)amide, which was synthesized as described previously (14), and bPTH-(1–84) was extracted from bo-

Production and purification of recombinant PTH analogs

Recombinant hPTH-(1–84) was prepared and purified as described previously (17). To facilitate the expedite production of different recombinant PTH analogs, we generated the plasmid pGTP-C2 from pGFP-1 (17). The factor Xa cleavage site of pGFP-1 was replaced by a thrombin cleavage site (Leu-Val-Pro-Arg) together with a three amino-acid (Gly-Gly-Thr) spacer that introduced a *KpnI* restriction site. Additional unique restriction sites were also introduced into pGTP-C2 to allow the rapid cassette replacement of various portions of the complementary DNA encoding human PTH-(1–84) (Fig. 1). The *KpnI*/*EcoT22I*, *EcoRI*/*HindIII*, and *HindIII*/*Eco81I* fragments of pGTP-C2 were then replaced by synthetic double strand oligonucleotides in which the

TABLE 1. Synthetic and recombinant peptides used in this study

Ligand	Abbreviated name	Binding to the PTH/PTHrP receptor	Binding to the C-PTH receptor
hPTH-(1–84)	PTH-(1–84)	+ + +	+ + +
[Leu^{8,18},Tyr^{34}]hPTH-(1–84)	mutPTH-(1–84)	+ +	+ + +
[Tyr^{34}]hPTH-(19–84)	mutPTH-(19–84)	−	+ + +
[Asn^{76}]hPTH-(39–84)	PTH-(39–84)	−	+ +
[Asn^{76}]hPTH-(53–84)	PTH-(53–84)	−	+
[Nle^{8,18},Tyr^{34}]bPTH-(1–34)amide	PTH-(1–34)	+ + +	−
hPTH-(44–68)	PTH-(44–68)	−	−
PTHrP-(37–74)		−	−
PTHrP-(109–141)		−	−
[Tyr^{36}]PTHrP-(1–36)amide	PTHrP-(1–36)	+ + +	−

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ATG codons for Met[8] and Met[18], and TTT for Phe[34] were changed to CTG (Leu[8,18]) and TAT (Tyr[34]), respectively. The resulting plasmid pGTP-Leu[8,18],Tyr[34] encodes the fusion protein hGH/[Leu[8,18],Tyr[34]]hPTH-(1–34). To produce [Tyr[34]]hPTH-(19–84) by cyanogen bromide digestion, the codon for Phe[34] in the HindIII/EcoB1I fragment of pGTP-C2 was replaced by TAT (Tyr[34]) to generate pGTP-Tyr[34]. The predicted nucleotide sequence of all plasmids was confirmed by nucleotide sequence analysis.

Both mutant hGH/PTH fusion proteins were prepared and purified from bacterial cultures as previously described (17), except that thrombin was used instead of factor Xa to cleave PTH-(1–84) from the fusion protein. To generate [Tyr[34]]hPTH-(19–84), [Tyr[34]]hPTH-(1–84) was digested with 50 mM cyanogen bromide in 70% formic acid according to standard protocols, followed by reverse phase HPLC purification to homogeneity using a 30–50% acetonitrile-0.1% trifluoroacetic acid gradient over 30 min at a flow rate of 1 ml/min. The predicted amino acid sequence and composition were confirmed for both peptides by amino-terminal amino acid sequence analysis and hydrolysis for amino acid composition, and by mass spectrophotometry, respectively.

Other chemicals and reagents

8-Bromo-adenosine-3',5'-monophosphate (8Br-cAMP) and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO); the heterobifunctional cross-linker sulfo-SMPB was obtained from Pierce Chemical Co. (Rockford, IL), and ^{14}C-methylated proteins (mol wt range, 14,300–200,000 daltons) were obtained from Amersham Life Science (Arlington Heights, IL). All tissue culture media, gentamicin, 0.05% trypsin-0.53 mM EGTA, and horse serum were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT).

Cell culture

The following cell lines were obtained from the indicated investigators: ROS 17/2.8 cells from G. A. Rodan (Merck, Sharp, and Dohme, West Point, PA); rat parathyroid (PT-r3) cells from M. L. Brandi (University of Florence, Florence, Italy) (18); SaOS-2 cells from A. H. Tashjian, Jr. (Harvard School of Public Health, Boston, MA); opossum kidney cells from G. J. Strewler (University of California, San Francisco, CA); UMR106–01 cells from J. T. Martin (St. Vincent's Institute of Medical Research, Fitzroy, Australia); YCC cells from J. J. Orloff (Yale University, New Haven, CT); MG63 cells from American Type Culture Collection (Rockville, MD); LLCPK₁ cells from S. M. Krane (Arthritis Unit, Massachusetts General Hospital, Boston, MA); and LLCPK₁ expressing the cloned rat PTH/PTHrP receptor (clone AR-B44) (19) from F. R. Bringhurst (Endocrine Unit, Massachusetts General Hospital, Boston, MA).

Monolayer cultures of each cell line were grown in Eagle's Minimum Essential Medium (MEM; SaOS-2), calcium-free MEM (YCC), DMEM (LLCPK₁, AR-B44, MG63, and OK), Ham's F-12 (ROS 17/2.8), or Ham's F-12-Dulbecco's Modified Eagle's Medium (1:1; UMR106, PT-r3) supplemented with 5–10% FBS and gentamicin (10 mg/liter) and were incubated in a humidified atmosphere of 5% CO₂-95% air at 37 C. Medium was changed every 2–3 days, and cells were subcultured weekly using 0.05% trypsin-0.53 mM EGTA. Cells for radioreceptor binding studies were plated 5–6 days before each experiment at a density of 1 × 10⁵ cells/well; medium was changed every other day and 18–24 h before each experiment. For some experiments, 8Br-cAMP, dexamethasone, and PTH analogs were added to the culture medium for the last 2 days of culture, with daily changes of the medium.

RRAs

MutPTH-(1–84), mutPTH-(19–84), bPTH-(1–84), and PTHrP-(1–36) were radioiodinated with Na[125]I (2000 Ci/mmol; New England Nuclear Corp., Boston, MA) by chloramine-T as previously described (20). Each radiolabeled peptide was purified by reverse phase HPLC using a gradient of 30–50% acetonitrile-0.1% trifluoroacetic acid over 30 min at a flow rate of 2 ml/min. Fractions containing the peak radioactivity were stored at −20 C until use.

Cells were rinsed once with 0.75 ml binding buffer [BB; 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5% heat-

inactivated horse serum, and 0.5% heat-inactivated FBS] and then incubated with 100,000 cpm [^{125}I]mutPTH-(1–84), [^{125}I]mutPTH-(19–84), [^{125}I]bPTH-(1–84), or [^{125}I]PTHrP-(1–36) in the presence or absence of different unlabeled peptides in a total volume of 0.25 ml for 4 h at 16 C. Unbound radioligand was then removed, and the cell monolayers were rinsed three times with 0.75 ml BB. Cells were lysed with 1 N NaOH, and the lysates were counted in a Micromedic 4/200 γ-spectrometer (ICN Biomedicals, Horsham, PA). For protein determinations, 10 μl cell lysate were measured by the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions using BSA as standard. Data are given as the mean ± SEM of at least two independent experiments performed in duplicate or triplicate.

Affinity cross-linking studies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

ROS 17/2.8 or PT-r3 cells were plated in 24-well plates at a density of 1 × 10⁵ cells/well and incubated as described above. After reaching confluency, the cells were incubated in the presence or absence of 3 × 10⁻⁴ M 8Br-cAMP for 2 days (unless stated differently) with daily changes of the medium. After rinsing the cells with BB buffered with 50 mM HEPES (pH 7.7; HBB) instead of 50 mM Tris (pH 7.7), 200 μl [^{125}I]mutPTH-(1–84) or [^{125}I]mutPTH-(19–84) (each ~200,000 cpm/well) were added in the absence or presence of increasing concentrations of unlabeled PTH-(1–34), PTH-(1–84), mutPTH-(19–84), or PTH-(39–84). After incubation for 4 h at 16 C, the cells were rinsed twice with HBB and twice with protein-free HBB. One milliliter of 0.1 mM sulfo-SMPB in protein-free HBB was then added to each well, and incubation was continued for 30 min on ice. Cross-linking was terminated by adding 1 M Tris-HCl, pH 8.0 (100 μl/well). After 30 min on ice, the incubation mixture was aspirated completely from each well, and the cells were lysed with 50 μl SDS-PAGE sample buffer [4% (wt/vol) SDS, 80 mM Tris-HCl (pH 6.8), 20% glycerol, and 550 mM 2-mercaptoethanol]. After incubation at 65 C for 30 min, 10 μl of each cell lysate were analyzed by electrophoresis through a polyacrylamide gel (5–20% gradient) and subsequent autoradiography with an intensifying screen at −80 C for several days, as indicated in the figure legends.

Results

Screening of clonal cell lines for C-PTH receptors

Preliminary radioreceptor studies were conducted with [^{125}I]bPTH-(1–84) that was labeled on tyrosine 43 by the chloramine-T method. Due to the concurrent oxidization of both methionines (positions 8 and 18), [^{125}I]bPTH-(1–84) binding to the common PTH/PTHrP receptor was minimal. Several clonal cell lines were initially screened, and significant binding of [^{125}I]bPTH-(1–84) was observed to ROS 17/2.8 cells (total binding, 7.1 ± 0.59%) and to PT-r3 cells (total binding, 6.1 ± 0.04%). Radioligand binding was completely inhibited by coincubation with PTH-(1–84) (10⁻⁶ M) and partially inhibited by PTH-(39–84) or PTH-(53–84) (both 10⁻⁶ M), whereas PTH-(1–34) (10⁻⁶ M) had no or little effect. Other clonal cell lines showed reduced levels of radioligand binding (UMR106 cells, 2.0 ± 0.03%; opossum kidney cells, 3.3 ± 0.37%) or no binding (YCC cells, 0.22 ± 0.04%; SaOS-2 cells, 0.24 ± 0.03%; MG63 cells, 0.20 ± 0.02%; LLCPK₁ cells, 0.24 ± 0.03%). As ROS 17/2.8 and PT-r3 cells demonstrated the highest specific binding of [^{125}I]bPTH-(1–84), all subsequent studies to characterize the C-PTH receptor were conducted with these two cell lines and with recombinant mutPTH-(1–84) and mutPTH-(19–84), because both ligands could be generated in unlimited quantities and high quality.
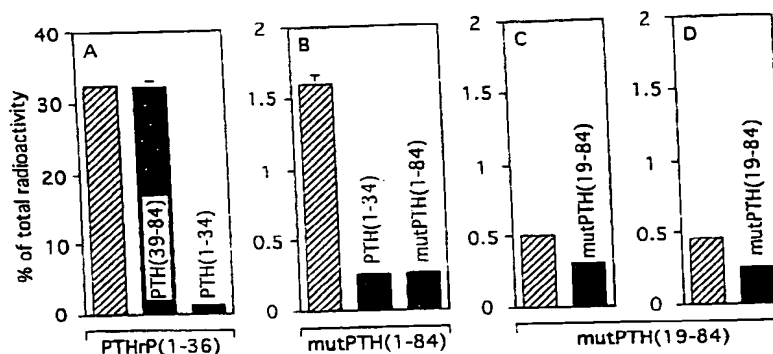
CHARACTERIZATION OF NOVEL PTH RECEPTOR

FIG. 2. Binding of radiolabeled PTH- (1–84) analogs and PTHrP-(1–36) to the PTH/PTHrP receptor. RRAs were performed as described in *Materials and Methods* with LLCPK₁ cells expressing the rat PTH/PTHrP receptor (clone AR-B44) (19) (A, B, and C) or with untransfected LLCPK₁ cells (D). Binding of [¹²⁵I]PTHrP-(1–36) (A), [¹²⁵I]mutPTH-(1–84) (B), or [¹²⁵I]mutPTH-(19–84) (C and D) was performed for 4 h at 16 C in the absence (☒) or presence (■) of different unlabeled PTH analogs (each peptide at 10⁻⁶ M). Data are expressed as the percent binding of total radioactivity and represent the mean ± SEM of at least three independent experiments performed in triplicate. Note the differences in scale for A.

RRAs using LLCPK₁ cells that express the cloned PTH/PTHrP receptor

To rule out that carboxyl-terminal PTH fragments specifically bind to the cloned PTH/PTHrP receptor, we first conducted radioreceptor studies with clonal AR-B44 cells (LLCPK₁ cells that express ~700,000 copies of the cloned rat PTH/PTHrP receptor/cell) (19) and different radiolabeled ligands in absence or presence of excess amounts of several PTH analogs. Using these cells, total radioligand binding was 32.4 ± 0.62% for [¹²⁵I]PTHrP-(1–36) and 1.6 ± 0.07% for [¹²⁵I]mutPTH-(1–84) (Fig. 2, A and B). Total binding of [¹²⁵I]mutPTH-(19–84) binding was even lower (0.5 ± 0.03%; Fig. 2C) than that observed with [¹²⁵I]mutPTH-(1–84), and it was indistinguishable from the binding observed with the parental LLCPK₁ cells (Fig. 2D). Nonspecific binding, assessed in the presence of the respective unlabeled peptide, was 1.3 ± 0.03% for [¹²⁵I]PTHrP-(1–36), 0.3 ± 0.01% for [¹²⁵I]mutPTH-(1–84), and 0.3 ± 0.01% for [¹²⁵I]mutPTH-(19–84).

RRAs using ROS 17/2.8 and PT-r3 cells

Radioreceptor studies with both cell lines gave similar results when using either [¹²⁵I]mutPTH-(1–84) or [¹²⁵I]mutPTH-(19–84) as radioligand (Figs. 3, A and B, and; 4, A and B). Interestingly, total and specific binding of [¹²⁵I]mutPTH-(19–84) was consistently about 2-fold higher than that of [¹²⁵I]mutPTH-(1–84). The amount of total radioactivity bound to ROS 17/2.8 cells was 5.1 ± 0.50% for [¹²⁵I]mutPTH-(1–84) and 11.3 ± 1.55% for [¹²⁵I]mutPTH-(19–84); nonspecific binding, assessed in the presence of 10⁻⁶ M PTH-(1–84), was 0.4 ± 0.10% and 0.6 ± 0.04%, respectively. Total binding to PT-r3 cells was 5.6 ± 0.52% and 9.3 ± 0.59%, respectively; nonspecific binding was 0.9 ± 0.13% and 0.4 ± 0.07%, respectively.

For both cell lines, the apparent Kd values were 20–30 nM for PTH-(1–84), mutPTH-(1–84), and mutPTH-(19–84), respectively, whereas PTH-(53–84) had an apparent Kd of more than 5000 nM (data not shown), and PTH-(1–34) and PTH-

FIG. 3. Radioreceptor studies with ROS 17/2.8 cells. Cells were incubated as described in *Materials and Methods* with [¹²⁵I]mutPTH-(1–84) (A), [¹²⁵I]mutPTH-(19–84) (B), or [¹²⁵I]PTHrP-(1–36) (C) in the absence or presence of increasing concentrations of PTH-(1–84) (■), mutPTH-(1–84) (O), mutPTH-(19–84) (□), PTH-(39–84) (●), or PTH-(1–34) (▽) for 4 h at 16 C. Data are expressed as a percentage of the total binding and represent the mean ± SEM of at least three independent experiments performed in duplicate or triplicate.
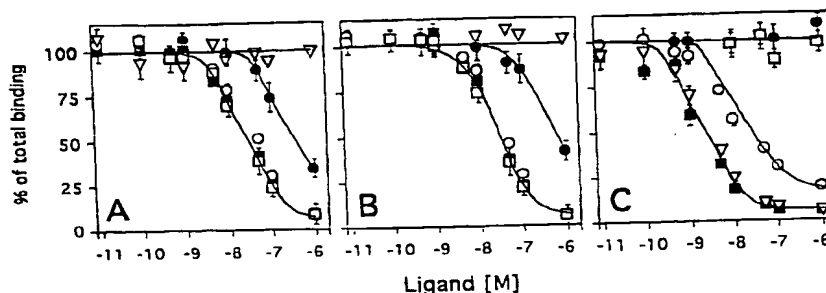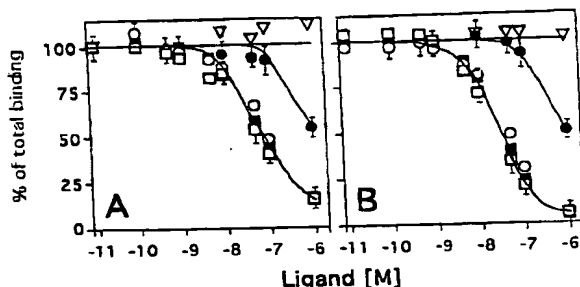
CHARACTERIZATION OF NOVEL PTH RECEPTOR

FIG. 4. Radioreceptor studies with PT-r3 cells. Cells were incubated as described in *Materials and Methods* with either [^{125}I]mutPTH-(1–84) (A) or [^{125}I]mutPTH-(19–84) (B) in the absence or presence of increasing concentrations of PTH-(1–84) (■), mutPTH-(1–84) (○), mutPTH-(19–84) (□), PTH-(39–84) (●), or PTH-(1–34) (∇) for 4 h at 16 C. Data are expressed as a percentage of the total binding and represent the mean ± SEM of at least three independent experiments performed in duplicate or triplicate.

(44–68) showed no inhibition of either radioligand (Figs. 3, A and B, and 4, A and B). PTH-(39–84) revealed a slightly higher binding affinity when tested with [^{125}I]mutPTH-(1–84) (apparent K_d, ~400 nM) compared to experiments performed with [^{125}I]mutPTH-(19–84) (apparent K_d, ~800 nM). Scatchard analysis of these data indicated that the C-PTH receptor is expressed in ROS 17/2.8 cells at about 320,000 copies/cell and in PT-r3 cells at about 180,000 copies/cell. Carboxyl-terminal or midregional PTHrP fragments, PTHrP-(37–74) and PTHrP-(109–141), showed no displacement of [^{125}I]mutPTH-(1–84) when tested with either cell line (data not shown).

When using [^{125}I]PTHrP-(1–36) (Fig. 3C), ROS 17/2.8 cells bound PTH-(1–84) and PTH-(1–34) with indistinguishable apparent K_d values of about 2 nM, whereas the apparent K_d value for mutPTH-(1–84) was about 10-fold higher (~20 nM). Neither mutPTH-(19–84) nor PTH-(39–84) inhibited binding of the radiolabeled PTHrP analog. Binding of [^{125}I]PTHrP-(1–36) to PT-r3 cells was negligible (total binding, 0.6 ± 0.03%; nonspecific binding, 0.5 ± 0.09%).

These studies, which are qualitatively summarized in Table 1, suggested that specific binding of radiolabeled mutPTH-(1–84) and mutPTH-(19–84) to ROS 17/2.8 and PT-r3 cells involves the 19–84 region, and the 1–34 region by itself does not interact with this novel C-PTH receptor. Our data furthermore imply that the presence of the common PTH/PTHrP receptor, which is expressed at about 80,000 copies/cell in ROS 17/2.8 cells (21) and at about 700,000 copies/cell in AR-B44 cells (19), does not contribute to the interaction between radiolabeled PTH-(1–84) analogs and the C-PTH receptor.

Regulation of C-PTH receptor expression

Homologous down-regulation of the common PTH/PTHrP receptor on ROS 17/2.8 cells was observed in response to PTH and PTHrP, whereas dexamethasone treatment increased the expression of this receptor (20, 21). We,

TABLE 2. Effect of PTH, dexamethasone, and 8 Br-cAMP on C-PTH receptor expression in ROS 17/2.8 cells

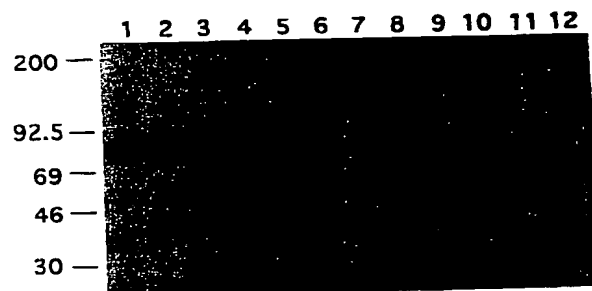| Treatment	Conc. (M)	% of control/100 μg protein
Control		100 ± 2
PTH-(1–34)	10^{-10}	124 ± 13
	10^{-9}	154 ± 13
	10^{-8}	153 ± 9
PTH-(1–84)	10^{-10}	132 ± 6
	10^{-9}	167 ± 14
	10^{-8}	166 ± 14
Dexamethasone	10^{-9}	112 ± 13
	10^{-8}	109 ± 12
	10^{-7}	122 ± 12
PTH-(39–84)	10^{-8}	105 ± 17
	10^{-7}	104 ± 9
	10^{-6}	108 ± 14
8Br-cAMP	3×10^{-4}	213 ± 12

FIG. 5. Affinity labeling of ROS 17/2.8 cells using either [^{125}I]mutPTH-(1–84) (lanes 1–6) or [^{125}I]mutPTH-(19–84) (lanes 7–12) and the heterobifunctional cross-linker sulfo-SMPB as described in *Materials and Methods*. Four days after reaching confluency, duplicate wells of ROS 17/2.8 cells were treated with medium alone (lanes 1, 2, 7, and 8) or for 24 h (lanes 3, 4, 9, and 10) or 48 h (lanes 5, 6, 11, and 12) with medium supplemented with 8Br-cAMP (3×10^{-4} M). After cross-linking and lysis of the cells with 50 μl SDS-sample buffer, 10 μl of each lysate were electrophoresed through a 5–20% gradient polyacrylamide gel. Autoradiography was performed at −80 C for 7 days. M_r standards (×10^3) are indicated.

therefore, studied first the effect of PTH and dexamethasone treatment on C-PTH receptor expression in ROS 17/2.8 cells and determined that PTH-(1–34) and PTH-(1–84) increased the specific binding of [^{125}I]mutPTH-(1–84) dose dependently (Table 2). Dexamethasone and PTH-(39–84) had no measurable effect, but treatment with 8Br-cAMP (3×10^{-4} M) increased radioligand binding by about 2-fold. The protein content of the wells with untreated ROS 17/2.8 cells was 217 ± 28 μg/well (mean ± SEM for three independent experiments), and similar to previous data (21), treatment with either reagent had no significant effect on cell proliferation or protein synthesis. C-PTH receptor expression in PT-r3 cells was not affected by treatment with either reagent (data not shown). These data suggested that C-PTH receptor expression is differently regulated in ROS 17/2.8 and PT-3r cells, despite otherwise indistinguishable binding characteristics for both cell lines.

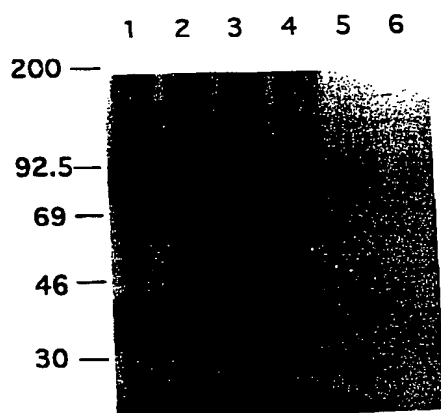CHARACTERIZATION OF NOVEL PTH RECEPTOR   4737

FIG. 6. Affinity labeling of 8Br-cAMP-treated ROS 17/2.8 cells using [^{125}I]mutPTH-(1–84) and sulfo-SMPB, as described in *Materials and Methods*. Control (lane 1) and affinity labeling in the presence of competing PTH-(1–84) (lane 2, 10^{-10} M; lane 3, 10^{-9} M; lane 4, 10^{-8} M; lane 5, 10^{-7} M; lane 6, 10^{-6} M) are shown. A 5–20% gradient polyacrylamide gel was used; autoradiography was performed at −80 C for 7 days. M$_r$ standards ($\times 10^3$) are indicated.

FIG. 8. Affinity labeling of 8Br-cAMP-treated ROS 17/2.8 cells using [^{125}I]mutPTH-(1–84) and sulfo-SMPB, as described in *Materials and Methods*. Control (lanes 1 and 2) and affinity labeling in the presence of competing PTH-(1–34) (lanes 3 and 4; 10^{-6} M), PTH-(1–84) (lanes 5 and 6; 10^{-6} M), mutPTH-(19–84) (lanes 7 and 8; 10^{-6} M), or PTH-(39–84) (lanes 9 and 10; 10^{-6} M) are shown. A 5–20% gradient polyacrylamide gel was used; autoradiography was performed at −80 C for 7 days. M$_r$ standards ($\times 10^3$) are indicated.
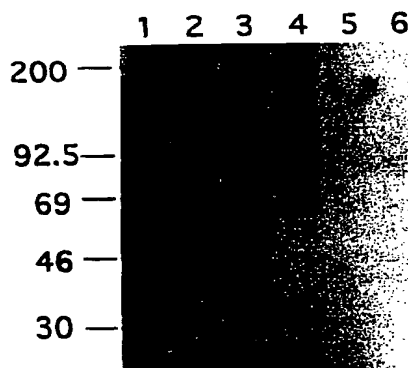

FIG. 7. Affinity labeling of PT-r3 cells using [^{125}I]mutPTH-(1–84) and sulfo-SMPB, as described in *Materials and Methods*. Control (lane 1) and affinity labeling in the presence of competing PTH-(1–84) (lane 2, 10^{-10} M; lane 3, 10^{-9} M; lane 4, 10^{-8} M; lane 5, 10^{-7} M; lane 6, 10^{-6} M) are shown. A 5–20% gradient polyacrylamide gel was used; autoradiography was performed at −80 C for 14 days. M$_r$ standards ($\times 10^3$) are indicated.
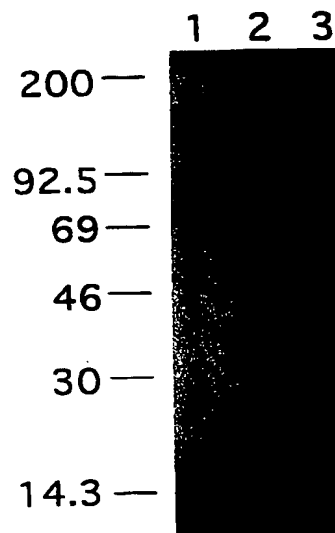
FIG. 9. Affinity labeling of the C-PTH receptor and the common PTH/PTHrP receptor. LLCPK$_1$ cells stably expressing the cloned PTH/PTHrP receptor (19) were photoaffinity labeled as described using ^{125}I-labeled [N$^{\alpha}$-(4-azido-2-nitrophenyl)Ala^1,Tyr^{36}]PTHrP-(1–36)amide (16) (lane 1). [^{125}I]mutPTH-(1–84) was cross-linked with sulfo-SMPB to 8Br-cAMP-treated ROS 17/2.8 cells, as described in *Materials and Methods* (lane 2, nonreducing conditions; lane 3, reducing conditions). A 5–20% gradient polyacrylamide gel was used; autoradiography was performed at −80 C for 7 days. M$_r$ standards ($\times 10^3$) are indicated.

Affinity cross-linking of the C-PTH receptor and SDS-PAGE of the ligand-receptor complex

To characterize the physico-chemical properties of the C-PTH receptor, affinity cross-linking experiments were first conducted with ROS 17/2.8 cells. As shown in Fig. 5, [^{125}I]mutPTH-(1–84) and [^{125}I]mutPTH-(19–84) were cross-linked to two protein bands in ROS 17/2.8 cells with mol wt of approximately 90 and 40 kDa, respectively (including the radioligand of ~10 kDa). Treatment of these cells with 8Br-cAMP for 24 h had no significant effect on C-PTH receptor
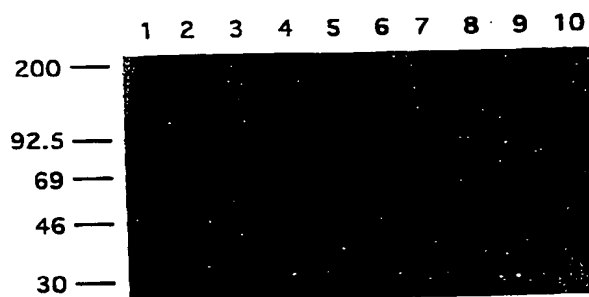
expression, but the prolonged treatment for 48 h revealed a 2- to 4-fold increase in the intensity of receptor labeling. Coincubation of cAMP-treated ROS 17/2.8 cells with [^{125}I]mutPTH-(1–84) and increasing concentrations of unlabeled PTH-(1–84) (10^{-10}-10^{-6} M) progressively diminished the intensity of C-PTH receptor labeling (Fig. 6).

either protein dose dependently and equivalently. Despite similar regulation and no apparent difference in ligand binding affinity, it remains to be seen whether both C-PTH receptor forms in ROS 17/2.8 cells are the products of two different genes, the result of a single, alternatively spliced message, or the result of alternative posttranslational processing.

The biological importance of this novel C-PTH receptor remains to be established, but it may well have important biological roles in osteoblast/osteoclast function, perhaps in mediating signals between these bone-specific cell populations (12). No second messenger has yet been identified that mediates the biological activity of carboxyl-terminal PTH fragments. Neither adenylate cyclase nor phospholipase C is stimulated by PTH fragments that lack the entire amino-terminal (1–34) region (10–12, 27, 28, and our own unpublished observations). However, carboxyl-terminal fragments of PTH-(1–84) appear to affect calcium up-take by SaOS-2 cells (29), and in ROS 17/2.8 cells, PTH-(8–84) stimulates membrane-associated PKC activity as efficiently as PTH-(1–34), PTH-(3–34), and PTH-(13–34) (28). This could indicate that the C-PTH receptor described herein is involved in the regulation of PKC activity. It is furthermore intriguing to speculate that carboxyl-terminal PTH fragments are involved in the feedback regulation of glandular PTH synthesis and secretion, because parathyroid-derived PT-r3 cells (18) express at least the approximately 90-kDa form of this novel C-PTH receptor.

If the C-PTH receptor is a single gene product, its size and abundance in the two clonal cell lines presented in this report should facilitate the isolation of its complementary DNA(s) through the same expression cloning techniques that were successfully used for molecular cloning of the common PTH/PTHrP receptor (24, 25).

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8. THE ROLE OF SITE-SPECIFIC RECOMBINATION IN EXPRESSION OF THE YEAST PLASMID 2 MICRON CIRCLE*

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The yeast plasmid 2 micron circle contains a specialized recombination system. There are two regions within the plasmid, each 599 bp in length, which are precise inverted repeats of each other and between which recombination readily occurs in yeast. This recombination, which requires a protein encoded in the plasmid itself at a locus designated FLP, establishes and maintains the coexistence within the cell of two distinct forms of the plasmid, designated A and B. These two forms differ structurally only in the orientation of one unique region with respect to the other. We provide evidence in this paper that FLP mediated recombination between the repeats is site specific. That is, the recombination does not occur throughout the inverted repeat but is limited to a specific sequence of less than sixty base pairs within the center of the repeat. In addition, the ability of a repeat to serve as substrate in this recombination is abolished by a four base pair deletion at the XbaI site in the middle of the repeat. This site lies at the center of an extended dyad symmetry and the possible role of this symmetrical feature in recognition by FLP protein is discussed. We previously proposed that FLP mediated recombination serves as a genetic switch. That is, we suggested that the transcriptional products of the two forms are different. Therefore, converting one form to the other by FLP mediated recombination would alter the composition of 2 micron circle transcripts in the cell. We have investigated this possibility by examining the transcriptional products of various Flp⁻ plasmids which are frozen in one orientation or the other or which contain large insertions at various locations within the genome. The results of this analysis, presented in this paper, argue that the transcriptional pattern of the plasmid is not altered by interconversion and thus that FLP mediated recombination does not serve to modulate transcription.

INTRODUCTION

The yeast plasmid 2 micron circle is a 6318 bp double stranded, circular DNA species present in most Saccharomyces strains at 50 to 100 copies per cell (12, 14). The plasmid has an unusual structure in that it contains two regions, each 599 bp in length, which are precise inverted repeats of each other and which separate the molecule into two unique regions of approximately equal size (12). In yeast recombination readily occurs between these two repeated sequences, which leads to the inversion of one unique region with respect to the other (1). As a consequence, 2 micron circle exists in yeast as a mixed population of two distinct plasmids which differ in the relative orientation of the two unique regions. These two forms have been variously designated A and B, XY' and XY, 14 and 23, or R and L (4). In mitotic cells,

detectable recombination between the repeat sequences requires a protein encoded in the plasmid itself, at a locus which we have designated FLP and which corresponds to the A coding region of Hartley and Donelson (5, 11, 12). In cells lacking FLP activity, no recombination between the inverted repeats of 2 micron circle is detected. Thus general mitotic recombination makes at most a minimal contribution to the interconverion of 2 micron circle.

No cellular phenotype has been convincing associated with the presence of 2 micron circles in yeast (4). Strains lacking 2 micron circles, designated [cir^o] strains, have been obtained either as natural isolates of standard laboratory strains or through competetive exclusion using high copy number hybrid 2 micron circle plasmids (9, 13, 16). Extensive analysis of isogenic [cir^o] and [cir$^+$] strains has failed to demonstrate any effect of the presence of 2 micron circles on growth rate or extent, mating ability or mating type interconversion, sporulation, or drug resistance (4). Nonetheless, 2 micron circle contains several genes in addition to FLP which are expressed in yeast. Two of these correspond to coding regions B and C and have been designated REP1 and REP2. Mutations in either of these genes prevent high copy number propagation of the plasmid. A third locus involved in high copy number propagation has been localized in the large unique region (M. Jayaram, Y-Y. Li, and J. Broach, manuscript in preparation). This locus, designated rep3, does not correspond to a coding region and is active only in cis. The location of the three 2 micron circle genes as well as the origin of replication of the molecule are indicated in Figure 1. Thus, although the plasmid does not provide a detectable cellular phenotype, a large portion of its genome is expressed in yeast, yielding activities apparently involved exclusively with its own maintenance.

On the basis of the transcriptional products of 2 micron circle in yeast, we previously proposed that FLP mediated interconversion in 2 micron circle is a requisite component of the expression of the plasmid (6). Specifically, we suggested that the transcriptional products of the two different forms of the plasmid are different. This is diagrammed in Figure 2. The polyA containing RNA species transcribed from 2 micron circles in yeast and the genomic locations from which each of them arise are indicated in Figure 2A. A scheme by which the all of these transcripts can be generated by cleavage of two primary transcripts, one from form A and one from form B, is shown in Figure 2B. In this model, transcription is initiated in the large unique region in both forms of the plasmid and continues through the inverted repeat into and through the smaller unique region. Since the smaller unique region of form A is in the opposite orientation with respect to the larger unique region than that of form B, the form A primary transcript will correspond to a different strand of the smaller unique region than that from form B. Thus transcription of form A and of form B yields different RNA species. Since interconversion would maintain both forms of the plasmid in a cell, each cell would possess both primary transcripts and thus a complete set of 2 micron circle mRNAs. Such a model would allow for the possibility that expression of the plasmid could be modulated through FLP mediated recombinational interconversion.

We have examined the extent to which the transcription model described in Figure 2 is correct by determining the in vivo transcriptional patterns of various hybrid 2 micron circle plasmids. These have included plasmids frozen in one orientation or the other or plasmids which contain insertions or deletions near the proposed primary promoter in the large unique region. The

FORM A
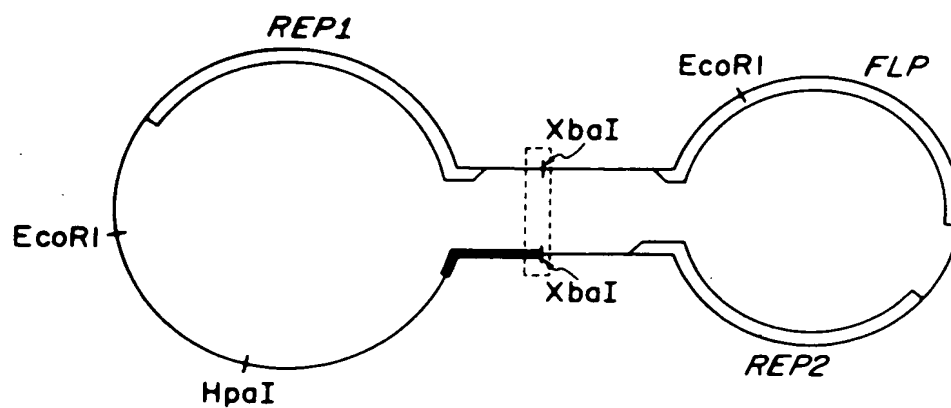
FORM B
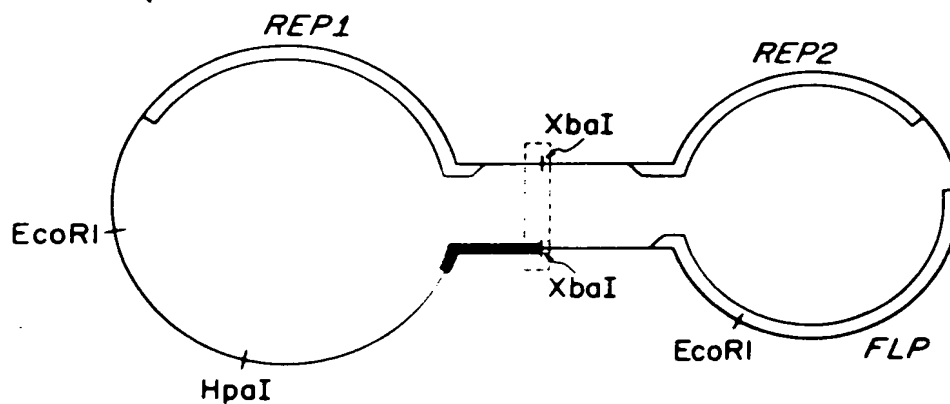
Figure 1.   The Yeast Plasmid 2 Micron Circle.

On a schematic diagram of the two forms of the 2 micron circle plasmid are indicated the locations and contents of the three identified plasmid genes (open bars; the taper in each indicates at the 3' end of the gene), the origin of replication (filled bar), and the region within which FLP mediated, site specific recombination occurs (dashed box). The circular portions of the figures represents unique sequences while the linear portions correspond to the inverted repeat sequences.
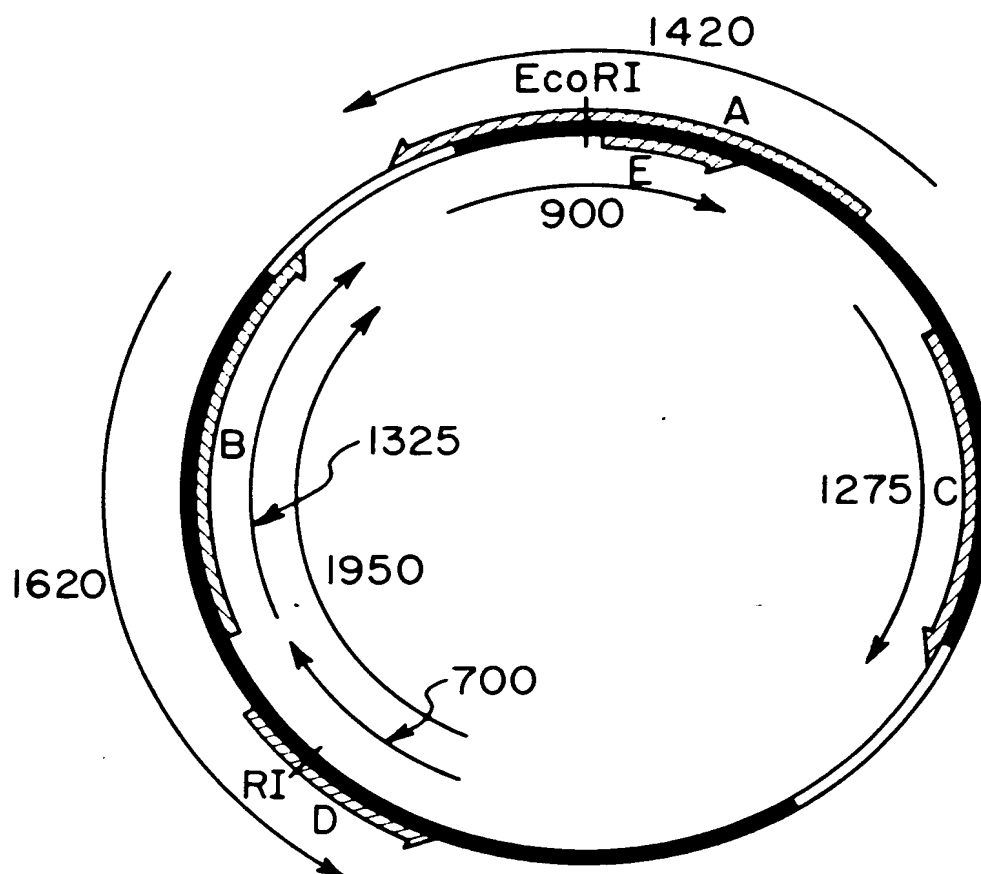
Figure 2.   Transcription of 2 Micron Circle.

A.   The positions from which a number of cellular polyA containing RNA species are transcribed in vivo from the 2 micron circle are indicated on a diagram of the A form of the plasmid.   The transcripts are identified by their lengths in bases.   The arrowheads lie at the 3' end of the RNA species and thus denote the direction of transcription of each of the species.   The locations and orientations of the five largest open coding regions of the plasmid are indicated by the hatched lines abutting the circle.   Regions A, B, and C, correspond to FLP, REP1, and REP2, respectively.   No phenotype has been observed for mutations within coding region D.   The inverted repeat regions of the plasmid are represented by the unfilled portion of the circle.

A form
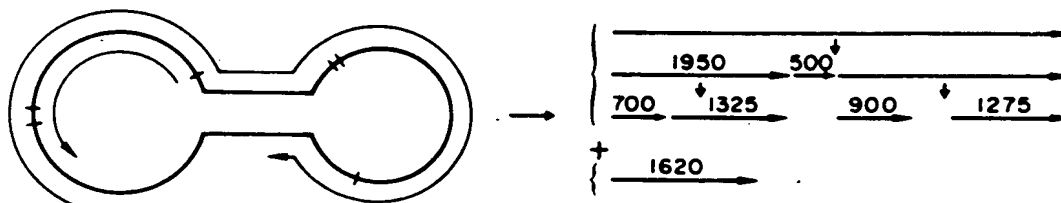
B form
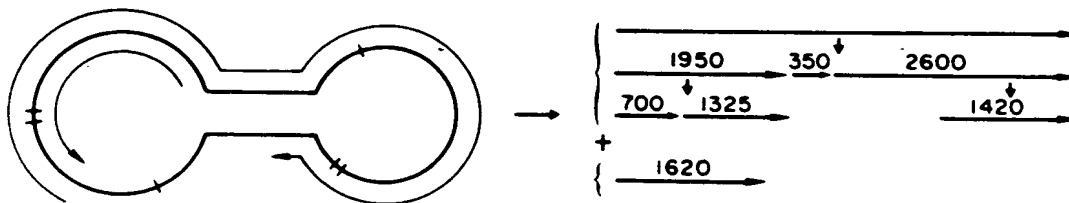
Figure 2.   Transcription of 2 Micron Circle.

B.   A scheme by which all two micron circle transcripts could be derived by transcription initiated at only two sites on the plasmid is shown. On the left the locations and orientations of postulated primary transcripts from the two forms of the plasmid are superimposed on dumbell shaped representations of the molecules. The EcoRI and HindIII sites are denoted by hatch marks.   On the right a pathway by which the hypothetical primary transcripts could be processed to yield all the observed 2 micron circle polyA RNA species is presented.

results from these initial studies are presented in this paper and suggest that the model as proposed is incorrect. The limitations of these experiments are discussed and other potential roles for FLP mediated recombination within 2 micron circle are proposed.

MATERIALS AND METHODS

Isolation of Tn5 Insertions.

Random insertions of Tn5 into plasmid CV20 were obtained by the procedure described by Ruvkun and Ausubel (15). E. coli strain JE5507 (man aroD arg lac spc str gal su⁺) harboring plasmid CV20 was infected with λ::Tn5 (λb221 rex::Tn5 cI857 Oam8 Pam29) at 32° and kanamycin resistant clones were recovered at a frequency of 10^{-5}. Since λ::Tn5 can neither lysogenize nor replicate in JE5507, kanamycin resistant clones can arise only by transposition of Tn5 to either the bacterial chromosome or to the resident plasmid. We reovered members of the latter class by isolating DNA from the pooled kanamycin resistant clones and using it to transform JE5507 to ampicillan and kanamycin resistance. Individual clones were retained, and the location of Tn5 insertion in indivdual plasmids was determined by appropriate restriction enzyme digestion of plasmid DNA isolated from small overnight cultures (3).

Plasmid Constructions.

Plasmid CV20 was derived from plasmid CV19, whose construction has been previously described (5), following propagation of plasmid CV19 in a [cir⁺] yeast strain and recovery of individual plasmids in E. coli. In one such plasmid, the large unique region of CV19 had been replaced, in the opposite orientation, with that from an endogenous plasmid. Thus, although CV19 has only a single EcoR1 site in the 2 micron circle moiety, CV20 has the normal two.

The constructions of plasmids HΔ24, XHinΔ1 and HinΔ14 have been previously described (5). We constructed plasmids XHinΔ2 and and HXΔ by cloning the appropriate XbaI restriction fragment of 2 micron circle onto a variant of plasmid CV03, in which the BamH1 site had been converted into an XbaI site. Plasmid XΔ2-1 was constructed by nuclease S1 digestion of XbaI digested CV7 DNA, followed by religation and recovery in E. coli.

Miscellaneous Methods.

E. coli and yeast transformation, preparation of DNA from yeast and E. coli, restriction analysis and Southern hybridization were performed as described previously (7). Isolation of polyA containing RNA from yeast, fractionation of RNA on denaturing agarose gels, transfer to diazotized (DBM) paper, and hybridization procedures were as before (6).

RESULTS

2 Micron Circle Recombination Is Site-Specific.

In order to investigate the mechanism of FLP mediated recombination in 2 micron circles, we isolated a set of insertion derivatives of a hybrid plas-

mid, CV20. Plasmid CV20 consists of pBR322, the LEU2 gene of yeast, and the entire 2 micron circle genome. The pBR322 plus LEU2 sequences are cloned into the EcoRl site in the small unique region (cf. Figure 1). Since the FLP gene spans this site, CV20 is flp and thus does not interconvert readily in [cir^o] strains. However, in [cir$^-$] strains recombination between the two inverted repeats on the plasmid readily occurs. Isolation of derivatives of CV20, each containing a random insertion of the bacterial transposon Tn5 into the 2 micron circle moiety of the plasmid, was accomplished using a procedure developed by Ruvkun and Ausubel and described in Materials and Methods. The locations of the Tn5 insertion in a number of isolates were determined by restriction enzyme analysis, and those plasmids containing an insertion within an inverted repeat were retained for analysis of FLP mediated recombination as described below.

The rationale of the procedure for determining whether FLP mediated recombination is site-specific and, if so, for localizing that site is diagrammed in Figure 3. In the center plasmid of part A is a diagram of the 2 micron circle moiety of a CV20 plasmid containing an insertion of Tn5 in the middle of an inverted repeat. For clarity, the pBR322 plus LEU2 sequences, which are present in the EcoRl site in the small unique region, have been omitted. Digestion of this plasmid with EcoRl plus BamHl yields three restriction fragments, the electrophoretic fractionation of which are schematically diagrammed in part B. Those fragments containing sequences homologous to Tn5 are indicated on the diagram. As shown in the figure, FLP mediated recombination occurring to the left of the Tn5 insertion in the plasmid I (site a) would generate plasmid II. Thus the cell in which such recombination occurs would contain a mixture of plasmids I and II. Digestion of this mixture of plasmids with EcoRl plus BamHl would yield five restriction fragments, as shown in part B, three of which contain Tn5 sequences. If on the other hand, FLP mediated recombination occurred only to the right of the insertion (at site b), then a Flp$^+$ cell in which plasmid I was introduced would generate a mixture of plasmids I and III. The digestion of this plasmid mixture with EcoRl plus BamHl would also yield five fragments, three of which contain Tn5 sequences. However, as is indicated in the figure, the restriction digestion pattern of the combination of plasmids I and III is clearly distinguishable from that of plasmids I and II. Finally, if FLP mediated recombination can occur anywhere throughout the inverted repeat, then a Flp$^+$ cell in which plasmid I was introduced would contain a mixture of all three plasmids. Digestion of this mixture of plasmids would yield four Tn5 containing fragments. Thus by determining the number of Tn5 containing restriction fragments present in a Flp$^+$ strain harboring a pCV20 plasmid with a Tn5 insertion in the inverted repeat, we can determine whether FLP mediated recombination is site specific. In addition, if recombination is site specific, then by assessing the relative sizes of these Tn5 containing restriction fragments, we can determine whether the site lies to the right or left of the Tn5 insertion.

The results of this analysis are presented in Figure 4. Individual derivatives of pCV20, each containing an insertion of Tn5 at the location indicated in part A, were used to transform isogenic [cir^o] and [cir$^-$ leu2] yeast strains to leucine prototrophy. Total cellular DNA was isolated from the transformants and digested with EcoRl plus BamHl. The digested DNA was fractionated by electrophoresis on agarose gels, transfered to nitrocellulose, and probed with labeled Tn5 DNA. The autoradiogram of the washed filter for

A.

BamHI   BamHI   BamHI

EcoRI   EcoRI   EcoRI   EcoRI

EcoRI   EcoRI   ←   EcoRI   a   b   →   b
                             a   EcoRI

Ⅱ   Ⅰ   Ⅲ

B.   Ⅰ   Ⅰ+Ⅱ   Ⅰ+Ⅲ   Ⅰ+Ⅱ+Ⅲ
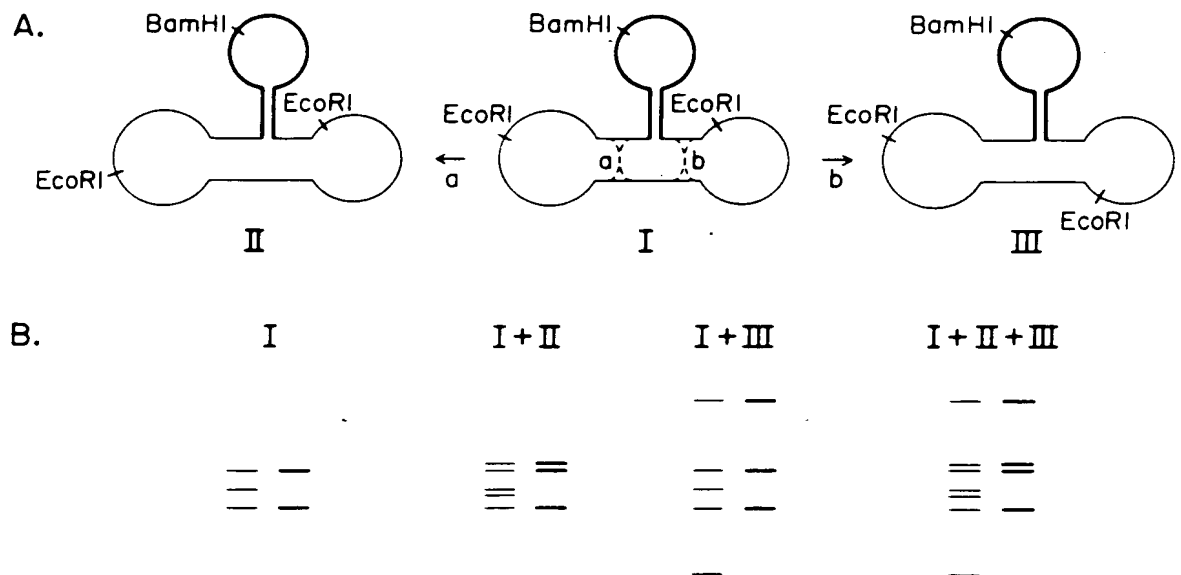
Figure 3. Scheme for Determining the Site of Recombination in 2 Micron Circle.

     In the center of the upper (A) portion of the figure is shown the B form
of 2 micron circle (thin line) containing a Tn5 insertion (heavy line) within
the inverted repeat (plasmid I). The locations of the EcoRl and BamHl sites
are indicated. In plasmid pCV20 used in this study, pBR322 plus LEU2 sequen-
ces are inserted as an EcoRl fragment in the EcoRl site in the right hand
loop. For clarity, this portion of the molecule has been omitted from the
figure. To the left and right are diagrammed the plasmids (II and III) which
would arise following recombination between the inverted repeats of 2 micron
circle, occurring either to the left or right of the Tn5 insertion, respec-
tively. In the lower (B) portion of the figure are schematic representations
of an agarose gel fractionation of restriction fragments which would result
from a BamHl plus EcoRl digestion of a mixture of the plasmids indicated above
each pair of tracks. For each set, the track on the left shows the position
of all the fragments which would arise from the digestion. The track on the
right shows only those fragments in the digestion which would contain Tn5
sequences.

two of these insertion derivatives is shown in part B. As previously described, no recombination products are observed after propagation of the two plasmids in the [cir^o] strain (track Flp$^-$). Thus, in the absence of the FLP gene product, recombination does not occur between the inverted repeats of 2 micron circle. However, after propagation of the plasmids in the [cir$^+$] strain, recombination products are clearly evident (track Flp$^+$). Significantly, in both cases only one additional Tn5 containing fragment is present. Thus, FLP mediated recombination does not occur throughout the inverted repeat but apparently is restricted to limited region within the inverted repeat. The pattern of Tn5 containing restriction fragments from plasmid pCV20::Tn5 #186, following propagation in the Flp$^+$ strain, indicates that the site of FLP recombination lies to the left of the Tn5 insertion in this plasmid. This is true for all of the plasmids containing a Tn5 insertion to the right of that in pCV20::Tn5 #186 (data not shown). Similarly, the pattern of Tn5 restriction fragments obtained from the Flp$^+$ strain containing pCV20::Tn5 #95 indicates that recombination occurs to the right of the Tn5 insertion in this plasmid. Again, this is true for all plasmids containing an insertion to the left of that in pCV20::Tn5 #95 (data not shown). Thus, the site of FLP mediated recombination is delimited to the region bracketed by Tn5 insertion #95 and #186.

We have obtained confirmation of the site specificity of FLP recombination as well as a further definition of the location and extent of the site by analysis of the ability of various hybrid plasmids, each containing different portions of an inverted repeat from 2 micron circle, to participate in FLP mediated recombination. In Figure 5 we present a diagram of various deletion derivatives of plasmid CV7. Plasmid CV7 consists of pBR322, the LEU2 gene, and the small EcoR1 fragment from the B form of 2 micron circle, which spans one of the inverted repeats as well as the 2 micron circle origin of replication. Various deletions extending into the inverted repeat sequence of this plasmid were constructed as described in Materials and Methods and the extent and location of the deletion in each of these derivatives is indicated in Figure 5.

The ability of each of these plasmids to serve as a substrate in FLP mediated recombination was assessed using two different assays. In the first assay we determined whether the deleted plasmid could recombine in yeast with resident, intact 2 micron circles, following transformation of [cir$^+$] leu2 strains. Those plasmids with a deletion extending into the inverted repeat from the left can only transform [cir$^+$] strains by recombining with the endogenous circles, since these deletions remove the 2 micron circle replication origin carried on CV7 (5). Thus, the ability of these plasmids to participate in FLP recombination could be recognized merely by determining whether or not they could transform a leu2 [cir$^+$] strain to leucine prototrophy at high frequency. However, for those plasmids with deletions internal to the inverted repeat or with deletions which enter the inverted repeat from the right, transformation is independent of the recombinational potential of the plasmid. Nonetheless, the extent to which each of the plasmids could recombine with a resident circle could be measured physically. This was accomplished by isolating DNA from a transformant, obtained with the plasmid in question, and determining by Southern analysis whether a species corresponding to a hybrid between the transforming plasmid and a resident circle could be detected.

(a)

(b)
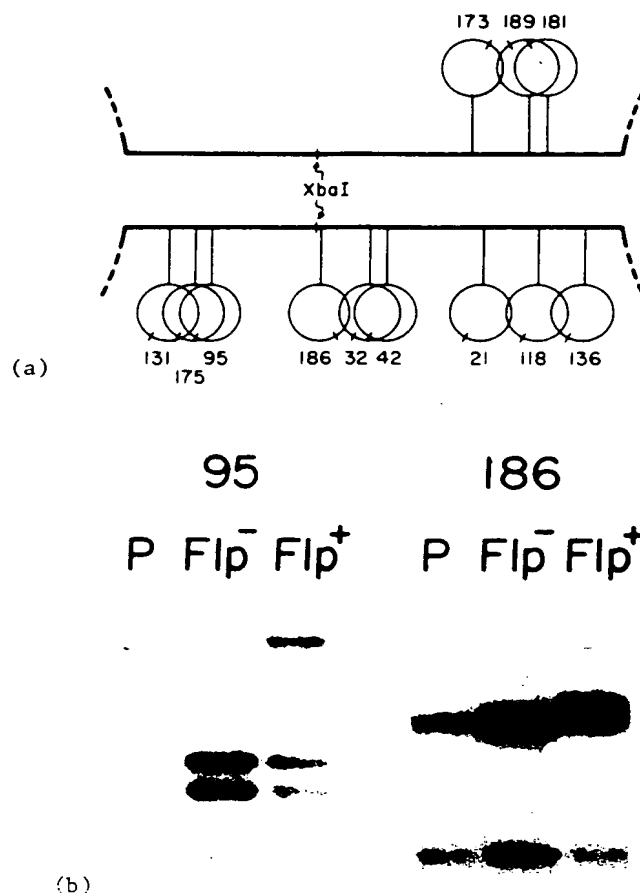
Figure 4.   Localization of the Site of 2 Micron Circle Recombination.

A.   The positions of individual Tn5 insertions used in this study are indicated as a composite of lollipop structures on a schematic representation of the two inverted repeats of 2 micron circle (heavy line).   The large unique region of 2 micron circle lies to the left.   The position of the XbaI restriction site in the inverted repeat is indicated.   In addition, the position of the single, assymmetric BamHl site in each of the Tn5 insertions is indicated by the slash in the circular portion cf the lollipop.

B.   After propagating plasmid CV20, containing the designated Tn5 insertion, either in the yeast strain DC04 [cir⁰] (track Flp⁻) or in strain DC04 [cir⁺] (track ⁻¹⁻⁺), total ⁻⁻⁻ was isolated from the strains, digested with EcoRl plus ⁻⁻⁻⁻⁻ ⁻⁻⁻⁻⁻⁻⁻⁻ ⁻⁻ ⁻ ⁻⁻⁻ agarose gel, transferred to nitrocellulose, and ⁻⁻⁻⁻⁻ with labelled ⁻⁻Tn5 DNA.   For reference purposes, purified plasmid ⁻⁻⁻ ⁻⁻⁻ ⁻⁻ ⁻⁻⁻⁻⁻⁻⁻ with BamHl plus EcoRl and fractionated on the same gel (lane P).   The resulting autoradiogram of the filter is shown. The upper band in track ⁻⁻⁻ for #186 is a doublet, which is clearer on a shorter exposure of the ⁻⁻ ⁻⁻⁻⁻⁻⁻⁻.
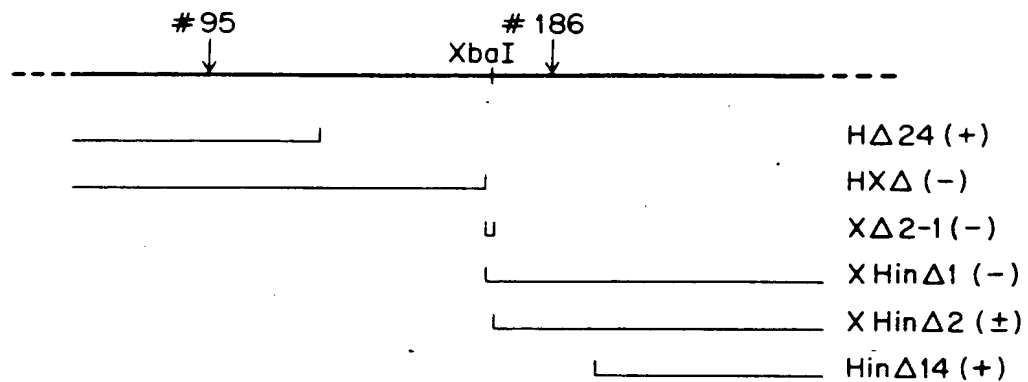
Figure 5.   Localization of the Site of 2 Micron Circle Recombination by
Deletion Analysis.

The heavy line at the top of the figure represents approximately 200 bp
spanning the XbaI site in the 2 micron circle moiety of plasmid CV7.   The
positions at which Tn5 insertions #186 and #95 map in the equivalent region of
plasmid CV20 are indicated.   Shown below the line are those sequences deleted
in the plasmids designated on the right.   In parenthesis next to each plasmid
designation is a "+" or "-" to indicate whether that plasmid retains or has
lost the site for recombination as determined by the assay described in the
text.

The second assay to determine the extent to which each deletion plasmid is a subtrate for FLP recombination was based on the recent observation that chromosomal integration of a segment of 2 micron circle spanning an inverted repeat causes a marked instability of the chromosome in which the insertion occurs (10). This instability is absolutely dependent upon FLP activity and the initial event in chromosome loss is a FLP-dependent recombination between the integrated plasmid and a resident 2 micron circle. Thus, by integrating each of the plasmids diagrammed in Figure 5 at leu2 (located on chromosome III) and then determining the frequency with which chromosome III is subsequently lost, we could could assess the extent to which each plasmid could participate in FLP recombination.

Both assays for the recombination potential of the various plasmids yielded equivalent, unequivical results, which are summarized to the right in Figure 5. Plasmids HΔ24 and HinΔ14 can participate in FLP mediated recombination to the same extent CV7, which contains an intact inverted repeat. All other deletion plasmids tested failed to display any activity as a substrate for FLP recombination. Thus, these results confirm that FLP recombination occurs at a specific site. In addition, we conclude that this site lies to the right of deletion HΔ24 and to the left of deletion HinΔ14, a site consistent with that defined by the Tn5 insertions. Finally, it is clear that the integrity of the sequences immediately spanning the XbaI site in the inverted repeat is crucial for FLP recombination, since a small deletion around this site completely abolishes recombination activity.

Recombination Within 2 Micron Circle Does Not Affect Expression of the Plasmid.

The Patterns of In Vivo Transcription From the A and B Forms of 2 Micron Circle Are Identical. We have previously proposed that transcription of the two forms of 2 micron circle yields different RNA species and that FLP mediated recombination promoted the interconversion of the two forms of 2 micron circle as a means of alternating between these transcriptionally distinct states (6). To test this hypothesis, we examined the in vivo transcriptional products of plasmids CV19 and CV20 in a [cir°] strain. Plasmids CV19 and CV20 are essentially identical except that the 2 micron circle genome carried on CV19 is in the A form while that of CV20 is in the B form. In both plasmids, however, pBR322 sequences interrupt the FLP gene. Thus both plasmids are flp and, as a consequence, each persists in its original orientation during propagation in [cir°] yeast strains. We isolated polyA containing RNA from [cir°] strains in which these plasmids were separately resident. These RNA samples were fractionated on denaturing agarose gels and plasmid specific transcripts were identified by hybridization with labeled 2 micron circle DNA following transfer of the RNA to DBM paper. These results are presented in Figure 6. As can be seen, the same RNA species complementary to 2 micron circle are present in both strains. Clearly evident in the exposure shown are the two relatively abundant transcripts from 2 micron circle -- namely, the 1325 and 1275 base RNAs, which are derived from REP1 and REP2, respectively -- and a larger trancript, which originates from within the pBR322 sequences carried on the two plasmids (unpublished observations). Longer exposures of the filter or hybridization using probes to specific regions of the 2 micron circle genome has failed to reveal any differences in the transcriptional products from the two plasmids. Thus the transcriptional products of CV19 and

CV19   CV20

2800

1325
1275
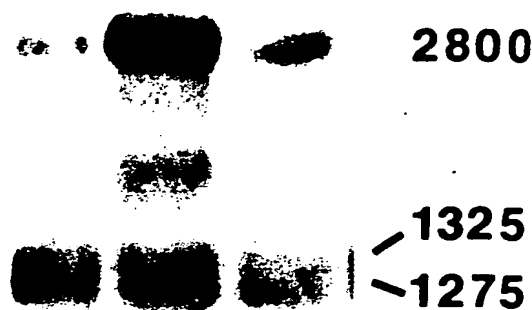
Figure 6.   Transcription Pattern from Individual Forms of 2 Micron Circle.

Plasmid CV19 consists of the entire A form 2 micron circle genome with pBR322 plus LEU2 sequences inserted into the EcoR1 site which lies in the middle of the FLP gene. Plasmid CV20 is identical to CV19 except that the 2 micron circle sequences are in the B form confiruration. Each of these plasmids were used to transform a [cir°] strain to leucine prototrophy and polyA containing RNA was obtained from representative transformants. Samples (10 μg) of the RNA from each transformant were fractionated separately or together (middle lane) on methyl mercury agarose gels, transfered to diazotized paper, and probed with labeled 2 micron circle DNA. The sizes of the predominant RNA species evident on the autoradiogram of the hybridized filter are indicated by length designations. The 2800 bp transcript apparently is initiated at an adventitious promoter site within the pBR322 moiety of the plasmid, extends into the 2 micron circle portion, and terminates within the inverted repeat at the normal site of termination of the FLP gene transcript.
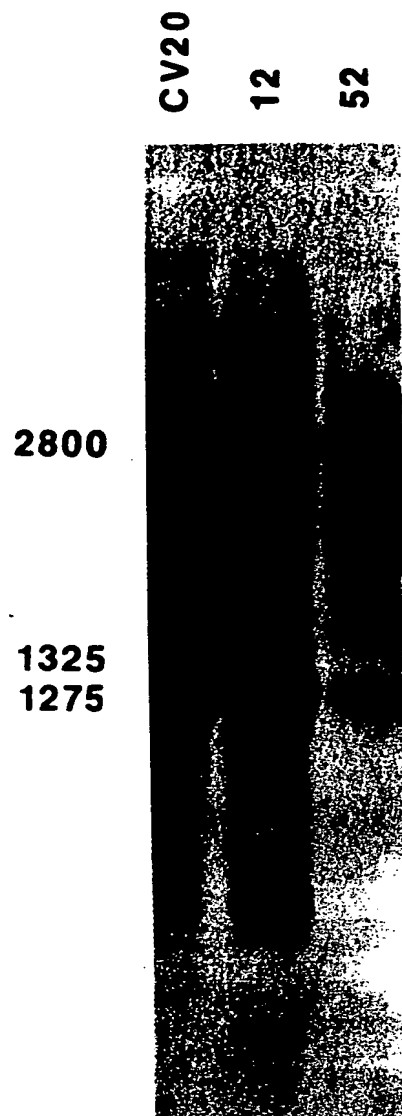
Figure 7.   Transcription Pattern of 2 Micron Circle Plasmids with Insertions in REP1.

PolyA RNA was isolated from a yeast strain in which was resident either plasmid CV20 or the CV20 derivative plasmid 12 or 52. Each of these latter plasmids contain a Tn5 insertion in the REP1 coding region.   The RNA was fractionated, immobilized and probed with labelled 2 micron circle DNA (ref 6).   The predominant species are indicated by size (in bases) designations.

CV20 Xho5

1900
1620

1325

Figure 8.   Transcription Pattern of a 2 Micron Circle Plasmid with a Deletion Spanning the Promoter of the 1900 Base Transcript.

PolyA containing RNA was isolated from a strain containing CV20 or a derivative of CV20, designated Xho5, in which sequences between the PstI site and the HpaI in the larger unique region of the 2 micron circle genome had been deleted. The RNA was fractionated, transfered, and probed with the nick translated 1314 bp HindIII fragment of 2 micron circle, which spans the 5' portion of REP1. The positions of migration of the three transcripts from this portion of the 2 micron circle genome are indicated.

CV20 are identical, suggesting that the transcriptional products of the two forms of 2 micron circle are also identical.

Insertions and Deletions in the Large Unique Region of 2 Micron Circle Have Little Effect on Plasmid Transcription. A second component of our hypothesis concerning 2 micron circle expression was that almost all transcription of the plasmid is initiated at a single site within the large unique region. To test the validity of this assumption, we have examined the effect on in vivo transcription of insertions within the large unique region, downstream from the proposed initiation site, and of deletions which remove this proposed initiation site. Plasmids CV20::Tn5 #12 and CV20::Tn5 #52 contain insertions of Tn5 within the REP1 coding region in the large unique region. These two plasmids as well as plasmid CV20 were introduced into a [cir°] yeast strain by transformation and polyA containing RNA was obtained from representative transformants. After fractionation on agarose gels and transfer to DBM paper, the plasmid specific transcripts were identified by hybridization with labeled 2 micron circle DNA. These results are shown in Figure 7. As is evident from the autoradiogram, the 1325 REP1 mRNA is absent from strains containing CV20::Tn5 #12 or #52. Less evident in the Figure, but clear in tracks probed with specific plasmid restriction fragments (data not shown), is the absence of the 1620 base transcript, which is transcribed from the same region but in the opposite direction as the REP1 RNA. However, except for a few minor, high molecular weight transcripts no other difference in the trancriptional products of the insertion containing plasmids versus the parent are apparent. Clearly, the 1275 base REP2 transcript is produced normally from both mutant plasmids. Thus, insertions within the REP1 gene in the large unique region fail to display any defect in the transcription of the small unique region.

By the procedure described in Materials and Methods we constructed a plasmid, designated Xho5, which is essentially identical to CV20 except that it contains a deletion in the large unique region which removes 400bp spanning the site corresponding to the 5' end of the 1950 base transcript (cf. Figure 2). Thus in the model we proposed for transcription of the plasmid, this deletion should have removed the principal 2 micron circle promoter. This plasmid was transformed into a [cir°] strain and the 2 micron circle specific transcripts examined as before. In the experiment shown in Figure 8 the RNA was probed with the 1314 bp HindIII restriction fragment from 2 micron circle, which covers the 5' portion of the REP1 coding region. As can be seen, plasmid Xho5 does not synthesize the 1950 base transcript. Nonetheless, the REP1 transcript is synthesized in near normal quantities, as is the 1620 base transcript. In addition, the normal contingent of transcripts from the small unique region are also synthsized from Xho5 (data not shown). Thus these results indicate that the 1950 base transcript is not an obligate precursor of the REP1 mRNA and that initiation in the large unique region is not a prerequisite for trancription of the small unique region.

DISCUSSION

By analysis of both insertion and deletion mutations within the inverted repeat region of the 2 micron circle, we have demonstrated that FLP mediated recombination occurs only at a specific site. Our analysis limits this site to a 65 bp region, although the actual site could be much smaller. Indeed.

the 4 bp deletion present in plasmid XΔ2-1 prevents this plasmid from participating in FLP mediated recombination.

The sequence of the DNA spanning the site of recombination is shown in Figure 9, within which are indicated the position of several relevant insertions and deletions. As is evident, this region of the plasmid is replete with symmetrical features. The XbaI site lies in the middle of a 22 bp region which is bracketed by a dyad symmetry that extends for 51 bp in either direction. Within each half of this dyad symmetry is a second dyad symmetry, so that this stretch of DNA could assume a cloverleaf structrue if it were single stranded. Viewed in a slightly different light, in the 100 bp region spanning the XbaI site is a sequence of 16 bp in length which is repeated, almost precisely, four times in alternating orientation. That this repeated sequence may play a role in the recombination event is suggested by the fact that this sequence also appears in the inverted repeat of Tn5, which under certain circumstances functions as a substrate for FLP mediated recombination (M. Jayaram and J.R. Broach, maunscript in preparation).

Whatever the actual recognition site is for the FLP recombination system, we can conclude that this site does not appear anywhere else in the yeast genome. We have recently shown that integration of the recognition site for FLP mediated recombination within a chromosome causes a marked instability of that chromosome (10). Therefore, if a site for FLP recombination existed in a chromosome, that chromosome would display significant instability. Since such an instability is not seen in normal yeast strains, we can assume that such a site does not exist.

Although the data presented in this paper demonstrate that FLP mediated recombination is initiated at a specific site, they do not address the mechanism of the recombination event. It is possible that recombination is initiated at or near the XbaI site by a single stranded nick. This could induce strand invasion, leading to the formation of a crossed strand intermediate which would be free to migrate throughout the inverted repeat before being resolve by appropriate strand scission. Evidence for the existence of such crossed strand intermediates, at least in meiotic cells, has been presented by Bell and Byers (2). As an alternative mechanism, FLP gene product could coordinately initiate and resolve the recombination event by, for example, a site-specific, double strand exchange. Additional experimental data will be needed to resolve this issue.

The function of FLP mediated interconversion of 2 micron circles is unknown. It is formally analogous to specialized recombination systems in procaryotic cells — such as G-loop inversion in phage mu (8, 17) and phase variation in Salmonella (18) — which function as genetic switches to permit alternate expression of different sets of genes. This analogy lent credibility to our previous hypothesis, based on the transcriptional pattern of 2 micron circle, that recombination functions as a genetic switch to interconvert transcriptionally distinct states of the plasmid. This hypothesis makes two testable predictions. First, the transcriptional products of the A form of the plasmid are distinct from those of the B form. Second, termination or inhibition of initiation of transcription in the large unique region should abolish transcription of the smaller unique region.

GTGCGTCTTC AGAGCGCTTT TGGTTTTCAA AAGCGGCTCTG AAGTTCCTAT ACTTTCTAGA GAATAGGAAC TTCGGAATAG GAACTTCAAA GCGGTTTCCGA
CACGCAGAAG TCTCGCGAAA ACCAAAAGTT TTCGCCGAGAC TTCAAGGATA TGAAAGATCT CTTATCCTTG AAGCCTTATC CTTGAAGTTT CGCAAAGGCT

XbaI

HΔ24   HXΔ-1 XΔ2-1   XHinΔ-2
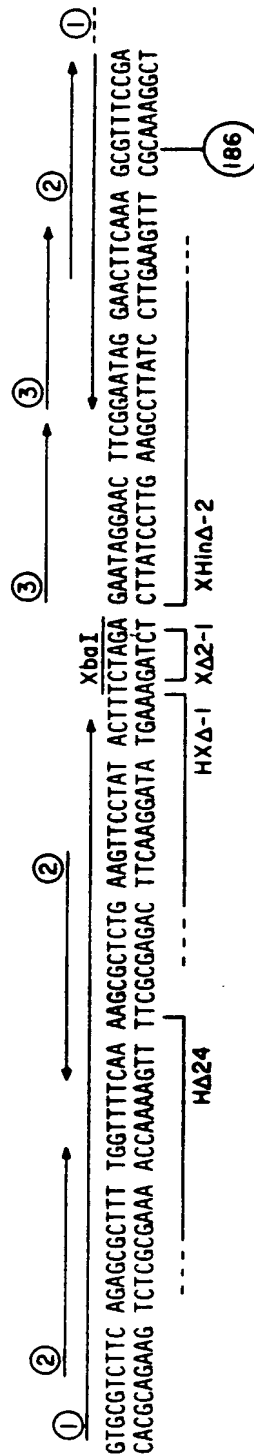
Figure 9. Sequence of the Site for Recombination in 2 Micron Circle.

The DNA sequence from part of the second inverted repeat of the A form of 2 micron circle (position 3891 to position 3990 in the numbering system of Hartley and Donelson, ref 12) is shown. Above the sequence are indicated regions of symmetry (numbered arrows) and the location of the XbaI restriction site. Below the sequence are indicated the location of Tn5 insertion θ186 and the regions deleted in several of the plasmids used in this study. The site required for recombination lies between the endpoint of deletion HΔ24 and position of Tn5 θ186.

In this paper we have presented results from transcription experiments which address these two predictions. First, we demonstrated that the in vivo transcriptional products of two hybrid plasmids, each containing the entire 2 micron circle genomes but frozen in opposite orientations, are identical. Additionally, we demonstrated that neither insertion of Tn5 sequences in the large unique region nor removal of the putative primary promoter of the plasmid abolished transcription of the small unique region. Thus, it would appear that our previous hypothesis is incorrect, and that an alternative explanation for FLP promoted recombination within 2 micron circle must be sought. It should be noted, though, that there is a caveat to the transcription experiments described in this paper. Namely, both pBR322 and Tn5 contain sequences which fortuitously function as promoters in yeast. As a consequence, it is possible that the 2 micron circle transcripts which should have been eliminated, due to the absence of recombination or to the insertion of Tn5, are being synthesized as a result of transcription initiated within one of these bacterial elements. Thus in spite of the data presented in this paper, the possibility that FLP mediated interconversion alters the transcriptional state of the plasmid cannot be definitively eliminated. Nonetheless, the transcriptional data presented in this paper is sufficiently suggestive that we are compelled to entertain other explanations for the function of FLP mediated recombination. One possible role for this recombination could be to ensure uniform segregation of the plasmid. That is, the recombination system could provide a mechanism by which catenated molecules arising following replication of the circular plasmid could be untangled. This possibility is being explored.

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9. PERIODIC SYNTHESIS OF HISTONE PROTEINS THROUGH THE CELL CYCLE OF Saccharomyces cerevisiae AS DETERMINED BY CENTRIFUGAL ELUTRIATION

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The methods of centrifugal elutriation, dual isotopic labelling, and Triton Acid Urea/SDS acrylamide gel electrophoresis have been used to study the synthesis of histone proteins through the cell cycle of the budding yeast, Saccharomyces cerevisiae. All three histone proteins studied, H2A, H2B, and H3, showed periodic patterns of synthesis. In each case the peak of histone protein synthesis substantially preceded that for the synthesis of DNA. The significance of the timing of histone gene expression, and its relationship to replication is discussed.

INTRODUCTION

The budding yeast, Saccharomyces cerevisiae, possesses a cell cycle similar to those of higher eukaryotes. It contains the same four major divisions of G1, S, G2, and M. Those differences which exist between Saccharomyces cerevisiae and higher forms are, for the most part, confined to mitosis. In the budding yeast, the nuclear membrane does not break down during mitosis (1), nor does the chromatin condense (2). However, the other stages of the cycle, including S, are very comparable to those of higher cells. Also, yeast chromatin is similar to that of higher cells (3,4). If yeast chromatin is digested with appropriate nucleases, the DNA fragments produced show a subunit structure similar to that of chromatin from higher eukaryotes (5,6,7). Like other fungi, yeast has a shorter DNA subunit repeat (6), which may reflect a shorter linker region (8).

Histone proteins H2A, H2B, H3, and H4 have all been characterized in yeast (9,7,10). They have been shown to be associated in normal nucleosomal structures with nuclear DNA and to have amino acid compositions comparable to those established from higher eukaryotes (11,12). However, the existence of a yeast H-1 protein has not been demonstrated clearly. Although an H-1-like protein has been observed (11), its properties are also similar to those of HMG (high mobility group) proteins (13,14,15,16,17,18).

In higher eukaryotes, the stage of the cell cycle defined by the period of DNA synthesis involves more than DNA replication. It includes the synthesis of histone and non-histone chromosomal proteins and their association with DNA to produce faithful and efficient replication of the chromatin unit (19). There is much evidence in higher eukaryotes for a coordination of DNA and histone synthesis. Many studies demonstrate that inhibiting the formation of one component hinders the synthesis of the other (20,21,22,23,24). In yeast, inhibition of protein synthesis with cycloheximide disrupts DNA synthesis during a short period at the beginning of S phase (25,26,27). This suggested a decreased temporal correspondence between histone synthesis inhibition and inhibition of replication and left open the possibility of continuous histone synthesis through the cycle. The data obtained by Moll and Wintersberger (19) argue for predominant, if not exclusive, synthesis of histones in S phase. However, the pulses used to measure synthesis of histone proteins were twenty minutes in duration, a full sixth of a cell cycle for many strains and equivalent to the entire period of S phase (1). More importantly, the methods used to obtain cell cycle specific fractions involved induction synchrony by alternating rounds of nutritional starvation and recovery (27,28,29). Such synchrony techniques may generate patterns of gene expression which are not observed when the cell cycle is studied by a non-stressful, cell-selection technique, such as centrifugal elutriation (30).

We report here a study of histone synthesis using centrifugal elutriation, a specific double isotopic labelling technique, and high resolution, Triton Acid Urea/SDS two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Strains

The diploid strain of Saccharomyces cerevisiae SKQ2n was used in all experiments described. It was obtained from Brian Cox (Oxford University) and has the following genotype:
    a/α; ade 1/+; +/ade 2; +/his 1

Chemicals

Purified yeast histone proteins were a gift from Court Saunders and Jim Davie at Oregon State University. DAPI (4´,6-Diamidino-2-phenylindol dihydrochloride) was obtained from Boehringer-Mannheim. All other chemicals were obtained from standard chemical sources. All radioactive labels were obtained from Schwarz-Mann.

Growth and Labelling of Cells

Cells were grown to midlog phase at 23°C on a rotary shaker. In all cases the medium used contained per liter: 6.7 g Yeast Nitrogen Base minus amino acids (Difco), 12.5 mg of each of the twenty amino acids naturally occurring in proteins except methionine, cysteine, and lysine, 20 g glucose, and 10 mg adenine and uracil. The doubling time of SKQ2n in this medium is 2 hours. In all cases, cultures used were 125 ml in volume, containing cells in mid-log phase (80 Klett units). The relative rates of synthesis for histones and other basic proteins were determined by a double-label procedure. Cells were labelled for 4 hours with [^{14}C] yeast protein hydrolysate (7.6 μCi/ml), then pulse-labelled for 10 minutes with [^{3}H] lysine (25 μCi/ml). Very heavily

labelled cells for the identification of histone spots on gels were obtained by labelling a 10 ml culture for 20 minutes with [^{14}C] yeast protein hydrolysate (12 µ Ci/ml). In all cases incorporation was halted by the addition of ice to the medium. The cells were immediately centrifuged at 5,000 rpm for 10 minutes at 0°C, washed once, and resuspended in ice-cold, distilled, deionized water.

Cell Cycle Fractionation and Fluorescent Staining

The procedure used for fractionation was described previously (30,31). Briefly, cells grown as described above were harvested on ice, resuspended in ice-cold, distilled, deionized water, sonicated to disrupt clumps and loaded at 2°C into a Beckman JE-6 rotor, spinning at 3000 rpm. After loading at 9 ml/min, 10 fractions (150 ml each) were collected and represent the entire cell cycle. Sodium chloride was added to each fraction to facilitate pellet formation, and the cells were collected by centrifugation.

The quality of separation obtained was determined by staining cells with the DNA specific stain DAPI, using the post-vital staining method of Williamson and Fennell (32).

Two-Dimensional Gel Electrophoresis

Triton Acid Urea/SDS two-dimensional gel electrophoresis was used to resolve basic proteins. 20 µl volumes of an RNase-DNase solution were added to cell pellets at 0°C – 4°C in 1.5 ml microfuge tubes (33). An additional 180 µl sterile, deionized water was added and the cell suspensions transferred to 3 ml sterile glass tubes, where oven baked glass beads (Glasperlen, 0.45 mm) were added to the meniscus. After lysis the extracts were transferred back into microfuge tubes and the beads washed twice with 200 µl of sterile, deionized water. Washes were pooled with the extracts and to each of these 0.5-0.6 ml volumes were added 1 ml of 50% TCA. The samples were allowed to precipitate on ice for ten minutes, then centrifuged for 2 minutes in a Beckman microfuge at 0°C. The resultant pellets were washed with acetone-HCl, then acetone precipitated (1 ml volume) for ten minutes on ice. After a 2 minute centrifugation, the resultant pellets were allowed to dry at 37°C for 1 hour. To these samples were added 25 µl AUT Sample Buffer and 25 µl acetic acid 6.7% in glycerol. Electrophoresis was performed as described previously (34,35).

Identification of Histone Spots on Gels

Co-electrophoresis of 10-20 µg of purified histone protein with total yeast protein extracted from 0.1 ml of an 80 Klett (midlog) culture of cells labelled for 20 minutes with [^{14}C] yeast protein hydrolysate (12.5 µCi/ml) was performed. This gave protein patterns on staining with Coomassie Blue in which yeast cell proteins were not discernable, but histone proteins were intensely stained. Autoradiograms of such gels showed the total yeast pattern after a two-week exposure. These autoradiograms were then used to locate spots to be cut out of gels. Spots were cut from the gels, solubilized for 48 hours using a toluene-based scintillant containing protosol (3.5%) and counted using a Beckman LS 8000 Scintillation Counter.

RESULTS

Resolution of the Rotor

We have used several methods to ascertain the accuracy of the separation

obtained by centrifugal elutriation (31,33). Biochemically, the peak of DNA synthesis (Fig.3) has been localized to fraction number four, a fraction which is composed almost exclusively of small budded cells (33). The morphologic composition of each fraction has been determined by post-vital staining of nuclei with the DNA-specific fluorescent dye DAPI (32). Data obtained from this method are shown in Table 1.

Table I

Cell Separation by Centrifugal Elutriation

Percentage of Cell Types

Fraction No.	Dead	Unbudded	Small Bud	Nuclear Migration	Doublet
1	26	66	1		
2	3	97			
3	2	29	69		
4		7	88	5	
5		13	62	18	7
6		16	24	45	15
7		10	39	28	23
8		8	24	26	42
9		8	17	24	51
10		9	18	26	47

The purity of fraction four discussed above is evident as is that for fraction two, which contains exclusively unbudded cells. Fraction three, however, represents a breakpoint between two morphological classes, containing 70% small budded and 30% unbudded cells. Subsequent fractions show varied distributions with fractions six and nine showing extreme enrichment for nuclear migrants and doublets, respectively. Dead cells are limited primarily to fraction one, which was therefore omitted from study.

Identification of Histone Proteins on Gels

The histones are low molecular weight, basic proteins. They are, therefore, best resolved by electrophoresis under acid conditions and high acrylamide concentrations. The system of choice was one described by Alfageme (35), which involves an acid extraction followed by a Triton Acid Urea first dimension. The addition of triton-X100, a non-ionic detergent, enhances the resolution of the histones, presumably by exploiting differences in hydrophobicity between otherwise similar molecules (35,36). Histones (H2A, H2B, H3, and H4), as well as a number of ribosomal proteins are well resolved (38). Histone H-1 was not identified in this system. Locations of the individual histone proteins were determined by co-electrophoresis of 10-20 μg of purified histone proteins with a low concentration of protein extract from cells which had been previously labelled with [14C] yeast protein hydrolysate to a high specific activity. This allowed identification of spots by comparison between stained gels, where only the histone standards were visible.

Histone Synthesis Through the Cell Cycle

Through the use of a specific double-labelling protocol and the Triton Acid Urea/SDS two-dimensional gel electrophoresis, it has been possible to determine

the nature of histone synthesis through the yeast cell cycle. The basis for such labelling and the analysis of plots obtained was described by Elliott and McLaughlin (33). Briefly, the plots represent the ratio of the synthetic rate (dA/dt) to accumulation (A) versus progression through the cell cycle (t). For exponential synthesis, this ratio is a constant and the plot is therefore a horizontal line. For periodic synthesis, there is a peak in the ratio corresponding to the period of synthesis, surrounded by regions of near zero ratio.

A pattern of periodic synthesis is observed for histones: H2A, H2B, and H3 (Fig. 1b,c,d). Histone protein H4 ran very near to the front of migration in this system. Accurate data could not be obtained. H2A and H2B exhibit periodic synthesis, with sharp peaks at fraction three. H3, similarly, shows periodic synthesis, with a peak in early fractions. For every experiment, ribosomal proteins run in the same gels were cut out, solubilized, and counted in exactly the same manner as the histone proteins. None of the ribosomal proteins showed periodicity (Fig. 1a), rather they displayed exponential pattern of synthesis observed for these proteins in earlier studies (37). A most interesting aspect of the histone synthetic data is the clear distinction in time between the synthesis of histones and the synthesis of DNA (Fig. 2). DNA synthesis peaks in fraction four in this system while all three histone proteins showed clear peaks of synthesis prior to fraction four.

FRACTION NUMBER
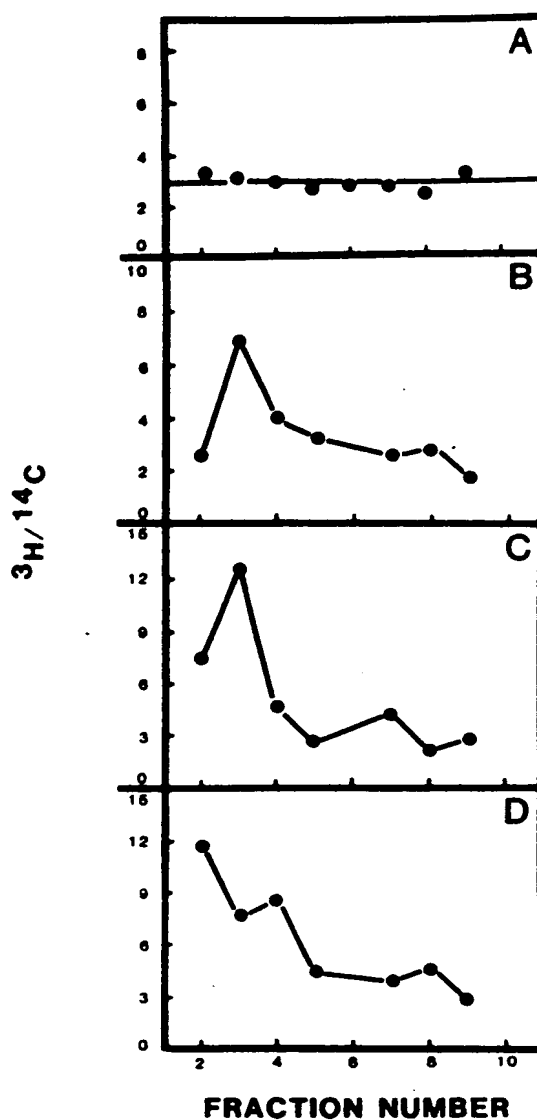
Fig. 1. Ratio of Pulse to Long-Term Radioactivity
(dA/dt ??) ??r??gh the cell cycle. Cells were labelled for 10 minutes. The cells were ?????? by elutriation and the synthesis of individual proteins determined as ??? ????. A corresponds to a representative ribosomal protein; B corresponds to ??st?ne mRA; C corresponds to histone H2B; and D corresponds to histone H3.
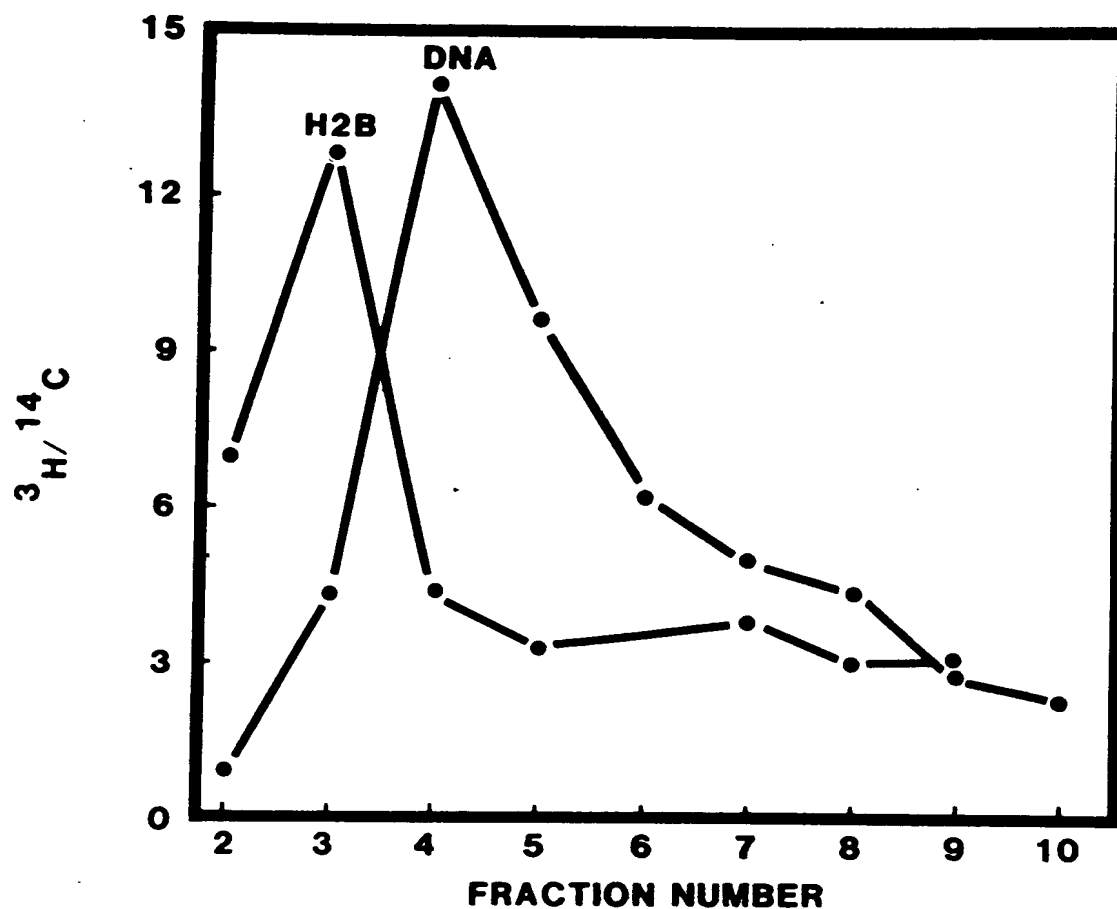
Fig. 2.   Ratio of Pulse to Long-Term Radioactivity
    (dA/dt/A) through the cell cycle for histone H2B and DNA. Cells were labelled and analyzed for histone synthesis as described in Fig.2 and for DNA synthesis as described by Elliott and McLaughlin (33).

DISCUSSION

Using elutriation, we have shown that the synthesis of histone proteins varies periodically through the cell cycle. This demonstrates the sensitivity of our methods to periodicities which are truly cell cycle specific. It is significant that these data are similar to those obtained using classic induction synchrony techniques (19). This suggests that although synchrony methods may generate periodicities which are independent of the cell cycle, they do not obliterate a set of periodicities which are truly of a cell cycle origin and required for cell division.

The high resolution of our cell selection method allows a precise localization of histone synthesis. We find that this synthesis does not persist through the whole of S phase, but is restricted to a period at the beginning of S phase. This point is significant in terms of a possible dependence of DNA synthesis on histone synthesis. The experiments of Hereford and Hartwell (26), as described in the INTRODUCTION showed that blocking protein synthesis early in S blocked DNA synthesis, while a later block in protein synthesis did not affect replication. If histone synthesis occurred throughout S, this finding would suggest the absence of a dependence relation. The restriction of histone synthesis to the beginning of S suggests a re-evaluation of this conclusion. Further, the coincidence of the period of required protein synthesis with that of maximal histone synthesis suggests the possibility that the required protein synthesis is mainly a requirement for histone synthesis.

Our observation that the peak of histone synthesis precedes that for DNA by approximately one-tenth of a cell cycle is consistent with those obtained by Hereford et al. (38). That study employed Northern analysis and in vitro translation of mRNA extracted from cells separated by elutriation. Taken together these studies indicate that transcriptional rather than translational control is important during the initiation period of histone synthesis. However, the interrelationship between DNA synthesis and histone synthesis is complex. Moll and Wintersbrger (19) concluded from experiments involving the use of hydroxyurea to block DNA synthesis that DNA synthesis was required for histone synthesis. We have shown that the inhibition of DNA synthesis leads to a rapid degradation of histone mRNA (38). On the other hand we find the highest level of histone synthesis occurs well before the peak of DNA synthesis. By the time the peak of DNA synthesis occurs histone synthesis has returned to near basal levels for H2A and H2B. This suggests that the mechanisms that control the amount of histone produced have to be fairly complex. They involve an initiation event for histone mRNA synthesis prior to the bulk of DNA synthesis, regulation of the amount of histone produced and a termination event which involves some post transcriptional control prior to completion of DNA synthesis (38). These mechanisms are under study.

We have described a method for biochemically sequencing events in a normal cell cycle. This method avoids abnormal growth conditions as well as physical and nutritional stresses. The sensitivity of the system has allowed precise characterization of the pattern of synthesis for a group of periodic proteins, the histones, and has allowed an acute positioning of these synthetic events within the cell cycle. These periodic molecular events in the cell cycle provide interesting insights into the nature of the mechanisms that control cell division and suggest that further study of the periodic molecular events of the cell cycle will be most profitable.

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10. CONTROL OF AMINO ACID BIOSYNTHESIS IN YEAST*

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In yeast, there is coordinate regulation of different amino acid biosynthetic pathways. In this paper we describe the isolation and characterization of 43 amino acid analog sensitive mutations which define four unlinked complementation groups, AAS101, AAS102, AAS103, and AAS104, two of which identify new genes involved in general control. We have shown that the AAS⁺ genes effect regulation at the mRNA level. Analysis of an aas101-complementing clone indicates that it (i) contains sequences that are repeated in the yeast genome, and (ii) codes for an RNA transcript that is derepressed under histidine starvation conditions. We have also demonstrated the existence of a transcript homologous to an open reading frame in the 5' flanking region of the HIS4 gene. A possible regulatory function for this transcript and/or polypeptide is discussed.

INTRODUCTION

In prokaryotes, functionally related genes are organized into common transcriptional units called operons which are controlled by a single regulatory region. In eukaryotes, however, no operons or polycistronic mRNAs have been identified (5, 10,14). Moreover, in several fungi it has been demonstrated, both genetically and biochemically, that, in addition to coordinate regulation of the different unlinked genes within a given pathway, there is also co-regulation of genes in completely different amino acid biosynthetic pathways (2, 3, 5, 11). This phenomenon is called "general control of amino acid biosynthesis" (4).

In Saccharomyces cerevisiae, it has been shown that starvation for a single amino acid causes derepression of the biosynthetic enzymes for all branched amino acids (aromatic and aspartate family), for the basic amino acids histidine, lysine and arginine, and also for serine and valine (9, 13, 19). (However, the extent of derepression of different enzymes within a given pathway may differ). The isolation of several regulatory mutations in this general control system has been reported. The mutations ndr1 (RH428), ndr2 (RH487) (for non-derepressing) and aas1, aas2 (for amino acid analog sensitive) are unable to derepress enzymes in different amino acid biosynthetic pathways (9, 13, 19). These mutations define three genes. It is postulated that these genes encode positive regulatory elements of the general control

system. Tra3 (for triazole alanine resistance) mutations have constitutive
levels of these same biosynthetic enzymes and it is presumed to be a negative
controlling element (19). The mechanism of action of these genes has thus far
remained elusive.

In this paper we present information which may bear on the molecular na-
ture of this general control system. We discuss: (i) the identification and
characterization of two new genes which are necessary for derepression of many
of these amino acid biosynthetic enzymes, and which may themselves be regu-
lated by conditions of amino acid imbalance, and (ii) the existence of a short
transcript (which has the capacity to code for a small polypeptide) in the 5'
flanking sequence of one of the genes under general control, the HIS4 gene.

We have attempted to saturate the yeast genome with amino acid analog
sensitive mutations to determine the number and mode of action of positive
regulatory genes involved in this general control of amino acid biosynthesis.
We have isolated 43 independent aas mutations which define four unlinked
complementation groups (AAS101, AAS102, AAS103, and AAS104), two of which
identify new genes involved in general control. These AAS genes effect amino
acid derepression by regulating mRNA levels. By screening a yeast genomic
library, genes which can complement different aas mutations have been cloned.
A preliminary characterization of several of these clones is discussed.

In addition, analysis of the 5' coding regions of several cloned yeast
biosynthetic genes subject to general control, HIS4, HIS3 and TRP5, indicates
that there are open reading frames preceding each of these structural genes.
It is striking that the position of the open reading frame preceding TRP5 is
almost identical to that of HIS4. We have demonstrated by both S1 protection
and primer extension experiments that a discrete transcript corresponding to
this 5' HIS4 region exists.

RESULTS

I. AAS Mutants

AAS Mutant Isolation

Forty-three independent aas mutants were isolated in the wild type strain
S288C by mutagenizing and screening for enhanced sensitivity to several amino
acid analogs. The rationale for this selection is that cells which become
sensitive to amino acid analogs may result from an inability to derepress the
amino acid biosynthetic enzymes which are necessary to overcome the delete-
rious effects of the analogs.

YEPD cultures of S288C (MATα) grown at 30° C were plated on YEPD medium
and irradiated with ultraviolet light to produce 50% killing. After two days
growth at 30° C, approximately 2×10^5 colonies were replicated to both
minimal and minimal + 0.75 mM 5-methyl tryptophan (5MT) medium. Forty-seven
5MT sensitive colonies were identified. Upon further screening, 43 of these
mutants giving a final mutation frequency of 2×10^{-4}), also exhibited sensi-
tivity to analogs of the end products of additional amino acid biosynthetic
pathways: 3-amino-1,2,4-triazole (AT), canavanine (can), and ethionine (eth),

analogs of histidine, arginine, and methionine, respectively. These putative general control mutations are designated aas for amino acid analog sensitivity, in accordance with the nomenclature of Wolfner et al. (19). About one-half of the mutants are also petites.

Genetic Analysis of the aas Mutants

Dominance. All of the aas mutations are recessive. Dominance was tested by crossing each of the aas mutants to an AAS$^+$ strain. The resulting heterozygous diploids show the phenotype of the AAS$^+$ parent when tested for amino acid analog sensitivity.

Complementation groups. The 43 aas mutations define four complementation groups called AAS101, AAS102, AAS103, and AAS104. The distribution of the mutations is: four alleles in aas101 (-1 to -4), 20 alleles in aas102 (-1 to -12, -14 to -21), 18 alleles in aas103 (-1 to -18) and one allele in aas104 (-1). These groups were determined by crossing the 43 aas mutants to previously isolated mutants which also show sensitivity to various amino acid analogs: ndr1-2 (RH428), ndr2-1 (RH487) (9, 13) and aas1 and aas2 (19). Representatives of the newly isolated aas mutations in the opposite mating type (obtained by crossing original mutants to wild type), were also tested in pairwise combinations with the original 43 aas mutants. Complementation was determined by scoring analog sensitivity on minimal + AT (15 mM) plates (see Table 1). These results indicate that the currently existing mutations define five amino acid analog sensitive complementation groups: I (AAS101), II (AAS102, NDR2, AAS1), III (AAS103, NDR1), IV (AAS104), and V (AAS2). Thus, two of the groups isolated in this study, AAS101 and AAS104, define two new genes involved in general control of amino acid biosynthesis.

Segregation. The aas mutations segregate as single mutations. Crosses of aas101-1, aas102-1, aas103-1, and aas104-1 to AAS$^+$ strains show 2:2 segregation for the aas mutation.

Linkage. Meiotic linkage data is consistent with the complementation results described above. Pairwise crosses between many of the aas mutants were subjected to tetrad analysis. Two classes emerged: (i) 101 x 102, 101 x 103, 101 x 104, 102 x 103, 103 x 104, 102 x 2, and 103 x 2; and (ii) 102 x ndr487, 102 x 1, and 1 x ndr487. The mutations paired from class (i) are unlinked to each other: PD (parental ditype) = NPD (nonparental ditype) for each cross. However, the mutations paired from class (ii) do not give any recombinants between the respective two mutations: all tetrads are PD (four AT sensitive spores/tetrad). This result indicates that the mutation pairs from (ii) are tightly linked, supporting the complementation test results which indicated that these mutations are in the same gene.

In addition, the AAS101 gene shows tight linkage to the URA3 gene on chromosome V.

Double mutants. Combinations of the aas mutations were constructed in haploid strains from the crosses discussed in the linkage section: 101-102, 101-103, 101-104, 102-103, 103-104, 1-2, and 103-2. All of the double mutation haploid strains are viable, and are sensitive to the amino

Table 1. Complementation of aas Mutations.

	aas101-1	aas102-1	aas103-1	aas104-1	aas1	aas2	ndr1	ndr2
aas101 (-1 to -4)	-	+	+	+	+	+	+	+
aas102 (-1 to -12, -14 to -21)	+	-	+	+	-	+	+	-
aas103 (-1 to -18)	+	+	-	+	+	+	-	+
aas104 (-1)	+	+	+	-	+	+	+	+
aas1	+	-	+	+	-	+	+	-
aas2	+	+	+	+	+	-	+	+

acid analogs.

    Homozygous diploids. The phenotype of the aas mutations in homozygous di-
ploids was also analyzed. The diploids aas101-1/aas101-1, aas102-1/aas102-1
and aas103-1/aas103-1 were constructed; they are sensitive to the same analogs
as the haploids. The 101, 102, and 103 diploids sporulate normally.

Analog Sensitivity of the aas Mutants

    The aas mutants exhibit enhanced sensitivity to various amino acid ana-
logs relative to their parent S288C. All 43 aas mutants were screened for
analog sensitivity by replica plating to minimal media supplemented with a
range of concentrations of AT, 5MT, can, or eth, at 23°, 30°, and 37° C (see
Table 2). The aas101 mutants are the most sensitive, and aas104 is the least
sensitive.

    Different mutations within a complementation group confer roughly the
same degree of sensitivity. However, in general, the petites are more sensi-
tive than the grande mutants within a given group. The largest variation in
sensitivity is seen in different aas102 and aas103 alleles. The most
sensitive grande mutations in these genes are aas102-14, -15, and -16, and
aas103-12, -15, -16, and -17.

    All the aas101, aas102, aas103, and aas104 mutants are temperature sensi-
tive in their analog response: increasing the temperature lowers the concen-
tration needed to inhibit growth. (For some of the analogs, S288C shows a
somewhat weaker ts phenotype.)

    In addition, an adenine effect was observed. The 43 aas mutants were
replica plated to minimal media supplemented with adenine as well as AT, 5MT,
can, or eth. The effect of adenine depends on the analog being tested: (i)
adenine increases the sensitivity to can or eth, (ii) adenine decreases the
sensitivity to AT, and (iii) adenine has no effect on 5MT sensitivity.

Revertants of the aas Mutants

    Almost all of the aas mutations give rise to spontaneous revertants as
measured by papillation when colonies are replicated to minimal + AT plates.
The most stable alleles are aas101-1, aas102-12, 102-19, 103-7, and 103-11.
Spontaneous revertants of several alleles of the aas mutations were sought by
growing 10 independent clones from each mutant in YEPD media and plating onto
minimal + 10mM AT to select for AT resistant clones. The frequency of
revertants was <1.6 x 10^{-8} for aas101-1, 9.8 x 10^{-6} for aas102-1, and 5.3 x
10^{-6} for aas103-1. Further testing on 5MT, can, and eth indicated a wide
range of resistance among the revertants to these additional analogs; some of
the revertants exhibited normal wild type resistance.

Aas Mutations Prevent Derepression of the HIS4 Gene

    Growth of his4C^{ts} strains. Aas101-1, aas102-1, aas103-1, and aas104-1
were crossed into a strain carrying a leaky his4 mutation, his4C-207^{ts}.
His4C-207^{ts} strains are unable to grow on miminal medium at 37° C but can grow

Table 2.   Amino Acid Analog Sensitivity of aas Mutants.

	5 MT				AT			Can				Eth			
	.5 mM	7.5 mM	1.0 mM	1.25 mM	10 mM	15 mM	20 mM	1.25 γ/ml	2.5 γ/ml	5.0 γ/ml	7.5 γ/ml	2.5 γ/ml	5.0 γ/ml	7.5 γ/ml	10.0 γ/ml
23°															
S288C	+	+	+	+	+	+	+	+	+	+	+/-	+	+	+	+
101's	+	+	+	+/-	-	-	-	+	+/-	-	-	+	+	+/-	+/-
102's	+/-	+	+	+	+/-	+/-	+/-	+	+	-	-	+	+	+	+
103's	+/-	-	+	+	+/-	+/-	+/-	+	+	+/-	-	+	+	+	+
104	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
30°															
S288C	+	+	+	+	+	+	+	+	+/-	-	-	+	+	+	+
101's	-	-	-	-	-	-	-	+/-	-	-	-	+	+/-	-	-
102's	+/-	-	-	-	-	-	-	+	+/-	-	-	+	+	+/-	+/-
103's	+/-	-	-	-	-	-	-	+	+/-	-	-	+	+	+	+
104	+	-	+/-	+/-	-	-	-	+	+	-	-	+	+	+	-
37°															
S288C	+	+	+	+	+/-	+/-	+/-	-	-	-	-	+	-	-	-
101's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102's	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
103's	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
104	-	-	-	-	-	-	-	+	-	-	-	+/-	+/-	-	-

on minimal media at 30° C because the cells compensate for the partially defective HIS4 enzyme by derepressing the histidine enzymes. However, the presence of any of the aas mutations (101, 102, 103, or 104) in a his4C-207^{ts} strain prevents growth on minimal media at 30° C. It can therefore be inferred that the wild type products of AAS101, AAS102, AAS103, and AAS104 are required for derepression of at least the HIS4C enzyme.

HIS4C enzyme levels. The effect of the various aas mutations on the level of HIS4C enzyme activity, histidinol dehydrogenase, was determined. The aas mutants and wild type cells were grown under repressing or derepressing conditions: (i) minimal + all 20 amino acids (min AA), (ii) minimal + histidine (min his), (iii) minimal (min), and (iv) minimal + 10mM AT (min AT). Wild type cells do not derepress on minimal media alone because the internal histidine pool is very high (5mM), and this pool does not get depleted given the basal wild type level of synthesis of the histidine biosynthetic enzymes (8). However, histidine starvation conditions can be achieved by growth of wild type cells in the presence of the analog AT. AT inhibits the sixth step in the histidine biosynthetic pathway. Wild type cells overcome this inhibition by derepressing the histidine enzymes, as well as other enzymes under general control. As Table 3 shows, the enzyme levels for wild type S288C are essentially the same when grown in min AA, min his, or min. However, on min AT, the levels of HIS4C enzyme levels are derepressed about six fold.

For the aas mutants, the levels on min AA, min his, and min, are the same, or slightly lower, if compared to S288C. However, when starved on min AT media, there is no derepression at all of HIS4C in the aas101, aas102, and aas103 strains; the aas104 strain consistently derepresses to only about one-half the maximal level of S288C. Thus, the aas strains are unable to overcome the AT inhibition. It can be concluded that all four AAS$^+$ genes are required for normal derepression of the levels of HIS4 enzyme activity to occur.

Table 3.   HIS4C Enzyme Assays of aas Mutants
Grown in Different Media

	AA	H	M	AT
S288C	1.0	1.2	1.2	6.3
aas101	.77	.80	.87	.40
aas102	.72	1.2	1.3	.55
aas103	.74	1.0	1.1	.74
aas104	.83	1.1	1.1	3.9

HIS4 mRNA levels. Northern blot analysis indicates that the aas mutations prevent derepression of the HIS4C enzyme by affecting mRNA levels. RNA was isolated from S288C, aas101, aas102, aas103, and aas104 strains, grown at 30° C on min AA, min his, min, and min AT. The RNA's were electrophoresed

on a 1.5% agarose formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labelled pMG1 (EcoRI-BglII fragment internal to the HIS4 structural gene) and YIp5 (pBR322 - URA3) probes. The URA3 gene was used as an internal standard to normalize for the amount of RNA in each lane. The mRNA levels for S288C and aas101 are shown in Figure 1. In the case of S288C, on minimal media supplemented with all the amino acids, a basal level of HIS4 mRNA is seen. A small derepression effect is observed on minimal or histidine media, whereas maximal derepression occurs when starved for histidine (min AT): the HIS4 mRNA is increased 5-10 fold. In the aas101 strain it is striking that little or no derepression of the HIS4 mRNA above basal level is detectable. Aas102 and aas103 show only intermediate derepression under starvation conditions, whereas aas104 shows the smallest effect (data not shown). These results suggest that the role of the AAS^{+} genes is to regulate the mRNA levels of the genes that they control.

Cloning the AAS Genes

Selection. We have constructed a yeast genomic library by ligating Sau3A partially digested DNA into the unique BamHI site of YEp24. The plasmid YEp24 contains both the yeast URA3 gene and part of the yeast 2μ plasmid, which per-mits episomal maintenance. This bank was used to clone genes which complement either the aas101 or aas103 mutation.

An aas101-1 ura3-52 strain (MP40A-8B) was transformed with the YEp24-S288C library. (Ura3-52 is a non-reverting allele). Ura^{+} transformants were selected in the presence of all 20 amino acids, pooled, and tested for aas101-complementing genes by growth on minimal + 10μM AT plates. Two inde-pendent AT resistant clones, called 101G and 101M, were identified from about 11,000 Ura^{+} transformants. The 101G insert is about 2.7 kb and the 101M insert is about 10 kb. Both of these clones also confer resistance to .75mM 5MT. In a second experiment, the library was used to transform an aas103-1 ura3-52 strain (MP42-2A). Two independent AT resistant clones were isolated from 8,000 Ura^{+} transformants. Both of these putative 103 clones were identical, and only one, 103R3, was used for further study. The 103R3 insert is about 5.5 kb.

The analog resistance property of these three clones resides on the plasmid in the transformed stains: spontaneous Ura^{-} segregants of 101G, 101M, and 103R3 simultaneously become aas^{-}. In the 103R3 clone, some AT sensitive Ura^{+} clones spontaneously arise, but these show altered restriction patterns of the YEp24 insert, suggesting the occurrence of some type of deletion within the plasmid.

Restriction maps. No similarities in restriction maps have been noted between 101G and 101M. This is consistent with our observation that no cross homology exists between the inserted sequences. However, the 101M and 103R3 clones have several contiguous restriction fragments in common. It is pos-sible that this common DNA sequence may complement both aas101 and aas103.

Homologous Genomic DNA. In order to confirm that the DNA cloned sequence represents a contiguous genomic sequence, rather than a cloning arti-fact, the following experiment was done. Genomic DNA from S288C was digested
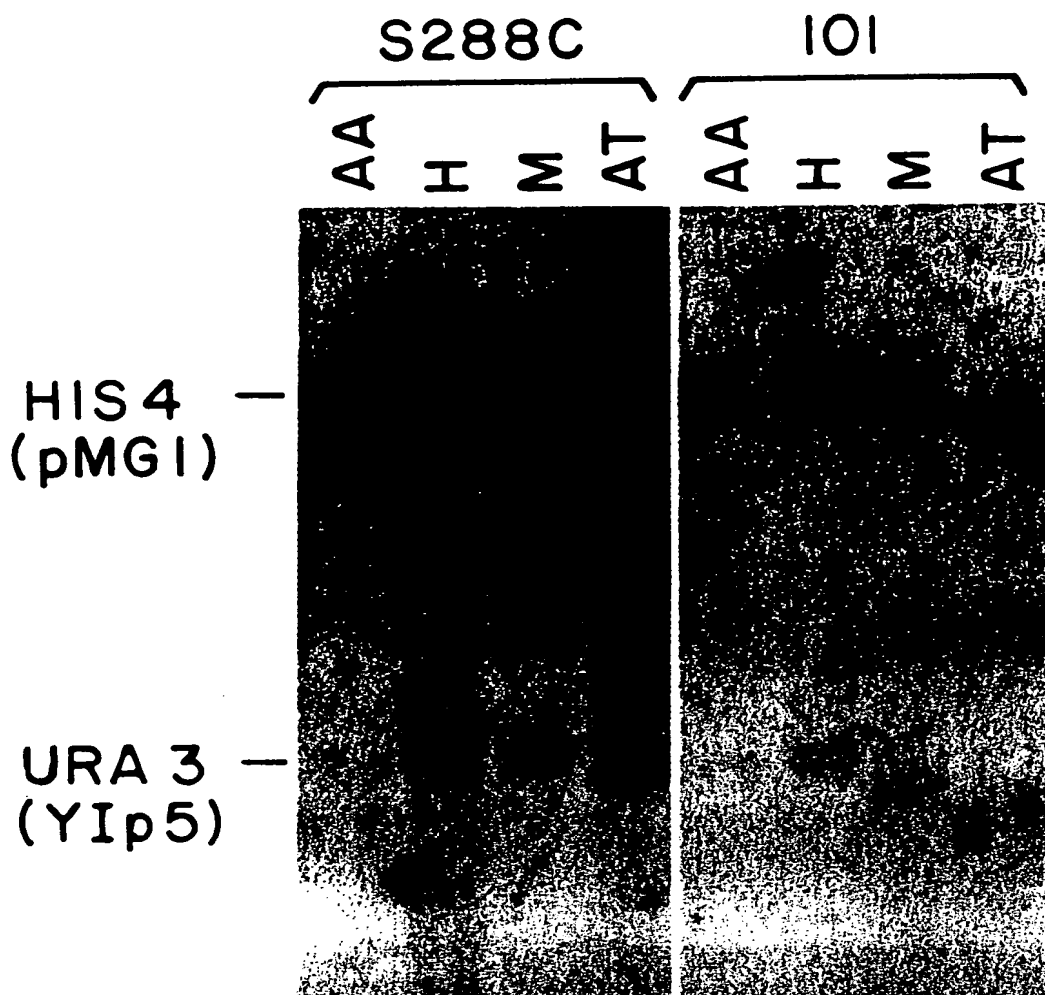
Figure 1.   Regulation of HIS4 mRNA levels in S288C and aas101-1.

RNA was prepared from wild type S288C and aas101-1 cultures grown to
O.D.$_{550}$ = 0.500 in minimal supplemented with all 20 amino acids (AA), minimal
histidine (H), minimal (M) and minimal + 10 mM aminotriazole (AT) media.
Extraction of RNA was by a modification of the procedure described in Sripati
and Warner (16). Approximately 15 ug of RNA was loaded per lane onto 1.5%
agarose formaldehyde gels. RNA was transferred to a nitrocellulose filter
(18). Bound RNA was hybridized with ^{32}P nick translated pMG1 (a 1.0 kb
fragment containing coding sequences in HIS4) and YIp5 (URA3). Hybridization
was in 50% formamide, 5XSSC, 45° C. The extent of hybridization with the URA3
probe was used to normalize for the amount of RNA within each lane.

with BamHI and PstI, electrophoresed on an agarose gel, and blotted onto nitrocellulose paper. The transferred DNA fragments were hybridized with ^{32}P-labelled BamHI-PstI fragment from 101G (see Figure 2). Figure 3 shows that a single band of 1.9 kb hybridizes strongly, as predicted if the cloned BamHI-PstI sequence is contiguous in the genome.

The BamHI-PstI fragment was used to probe a variety of genomic digests with restriction enzymes which fail to cut within the insert. As expected, only one band hybridized strongly in each digest. Surprisingly, however, several other weakly hybridizing bands are apparent. Many of these bands are smaller than the strongly hybridizing bands, indicating that they are not due to partial digestions. Therefore, sequences contained within the BamHI-PstI fragment are cross homologous to other sequences in the genome.

RNA Homologous to 101G. The level of RNA in various strains corresponding to the 101G clone was determined by Northern blot analysis. RNA was isolated from S288C, aas101-1, aas102-15, aas103-15, and aas104-1 strains grown at 30° C on min AA, min his, min, and min AT. The RNAs were electrophoresed on a 1.5% agarose formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labelled SphI-SphI fragment from 101G and YIp5 (pBR322-URA3). The URA3 gene was used as an internal standard to normalize the amount of RNA in each lane.

The S288C levels of 101G RNA are roughly equivalent on min AA, min his, and min (see Figure 4). However, under conditions of his starvation, min AT, the 101G mRNA level is very greatly increased. Therefore, the gene homologous to the 101G clone is itself being derepressed under conditions of histidine starvation. This derepression of the 101G transcript is also observed in the aas101-1, aas102-15, and aas103-15 strains, and to a lesser extent in the aas104-1 strain. This result implies that derepression of the 101G mRNA itself is not dependent upon the AAS101$^+$, AAS102$^+$, or AAS103$^+$ genes (and also probably the AAS104$^+$ gene).

II.   5' HIS4 Transcript

5' Open Reading Frames

Examination of the nucleotide sequences of several yeast genes under general amino acid control, HIS4, TRP5, and HIS3, reveals the existence of a sequence which contains an open reading frame upstream from the transcriptional start site of these genes (see Figure 5). In the HIS4 gene, this open reading frame is 198 basepairs (bp); it starts with an ATG at position -383 and ends with an inframe TGA at position -185. If this region is transcribed and translated it has the capacity to encode a polypeptide of 66 amino acids. It is striking that the position of the open reading frame preceding TRP5 is very closely coincident with that of HIS4, -355 to -185. These upstream regions may be candidates for regulatory elements involved in the control of their linked structural genes.

Nuclease Mapping

To determine the possible involvement of this 5' open reading frame in

Restriction Map of 101G

101G = 2.7 Kb insert
— Cloned DNA
— Vector DNA
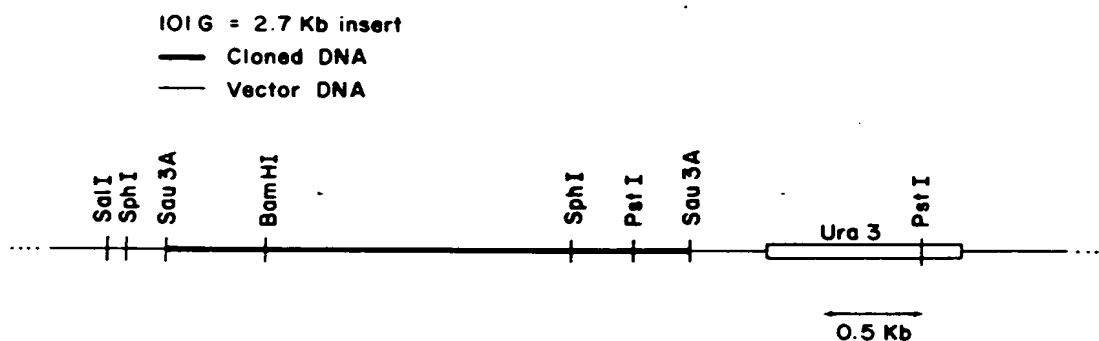
Figure 2.   Restriction map of 101G.

The Sau3A sites indicated in the figure are not unique, but rather indicate the limits of the inserted genomic DNA fragment. BglII, ... ,HindIII, SalI, SmaI and XhoI do not cut within the insert.
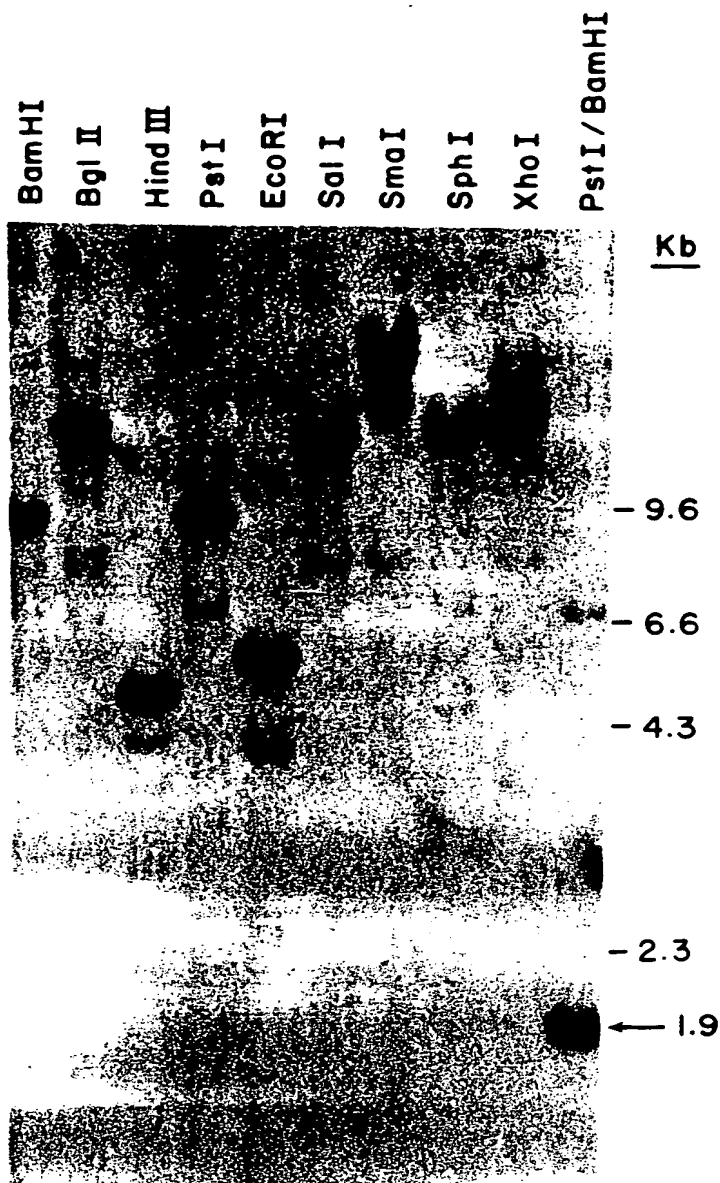
Figure 3.   Southern blot of S288C genomic DNA probed with the BamHI-PstI fragment from 101G.

S288C DNA was digested with the restriction enzymes indicated and electrophoresed on a 0.8% agarose gel. DNA was transferred to a nitrocellulose filter according to the method of Southern (15). The filter-bound DNA was hybridized to the nick-translated (12) 1.9 kb BamHI-PstI fragment from 101G (see Fig. 2). Hybridization was carried out at 65°C in 5XSSC.
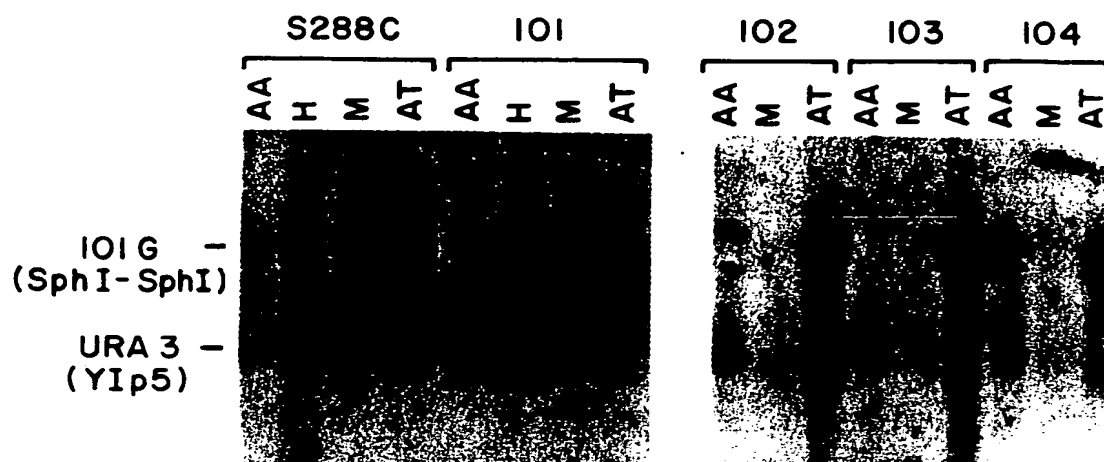
Figure 4.   Regulation of 101G encoded RNA levels in wild type
and aas mutant strains.

RNA was prepared from wild-type S288C, aas101-1, aas102-15, aas103-15,
and aas104-1, electrophoresed, and blotted as described in Figure 1.
Nitrocellulose bound RNA was hybridized with ^{32}P nick-translated DNA (12) from
the SphI-SphI fragment of 101G and with YIp5 (URA3). Hybridization conditions
were as described in Figure 1. The extent of hybridization with the URA3
probe was used to normalize for the amount of RNA within each lane.

HIS 4

(-331)   (-187)
Hinfl   Hinfl

-383   -185   -40   +I   +60
                                        TATA

RNA ····—————————————····►
      AUG   UGA   AUG

TRP 5

-355   -185   -40 +I +45
                                  TATA

RNA ····—————————————··►
        AUG   UGA   AUG

HIS 3

-252   -94 -40 +I +45
                  TATA

RNA   ····—————————————··►
           AUG   UGA   AUG
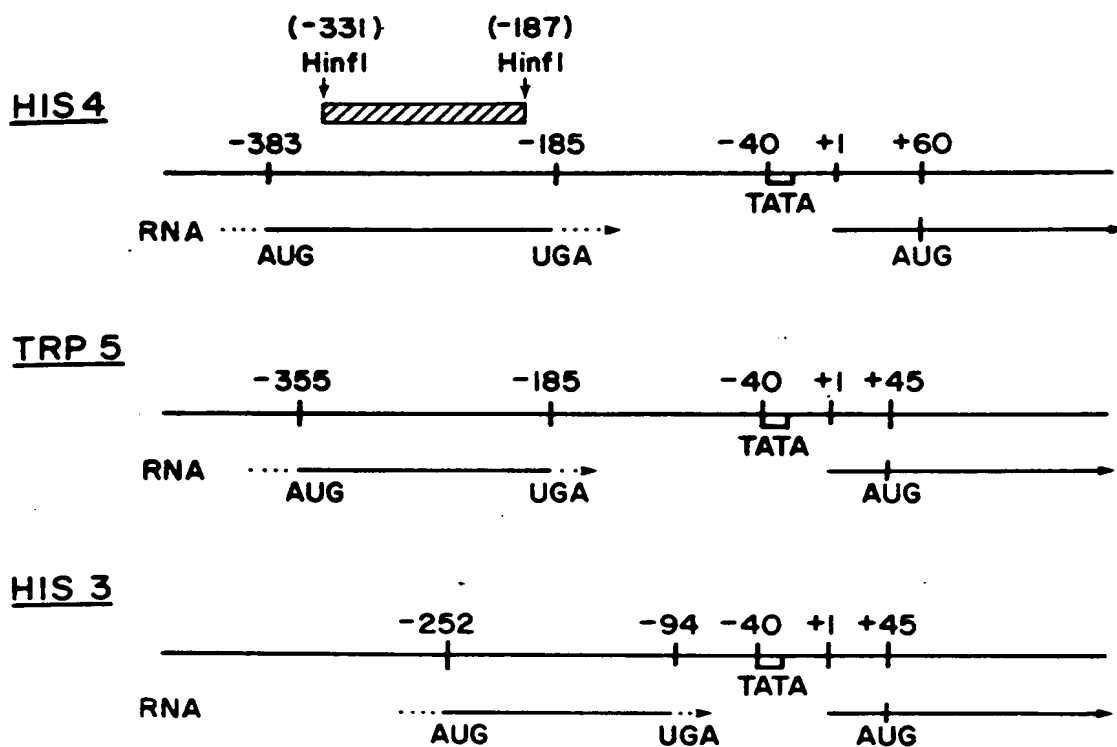
Figure 5.   Comparison of the yeast 5' flanking regions of
HIS4, TRP5 and HIS3.

The 5' regulatory regions are drawn to scale relative to the transcrip-
tion initiation sites (+1) for their respective structural genes.   The
positions of the open reading frames are indicated by the solid black lines;
the boundaries are delineated by ATG and TGA codons.   Positions of potential
TATA boxes are included for illustrative purposes.   The 148 bp HinfI fragment
used in the S1 protection and the primer extension experiments is indicated.
The sequence data is from P. Farabaugh and G. R. Fink, pers. comm. and (17,
20).

HIS4 gene expression, we first ascertained that this region was transcribed. A 148 bp HinfI fragment (see Figure 5), extending from -331 to -187 (internal to the sequences encoding the presumptive polypeptide), was isolated from a polyacrylamide gel and labelled at the 5' end with $[\gamma-^{32}P]$ ATP. The labelled DNA was denatured and separated into fast and slow migrating strands on a 5% polyacrylamide gel. Total yeast RNA was incubated with each of the strands to allow homologous regions to anneal. This was followed by digestion of single stranded regions with nuclease S1. The protected annealed complexes were resolved on an 8% denaturing polyacrylamide gel and autoradiographed. As seen in Figure 6, there is specific protection from S1 digestion of the fast migrating DNA strand (lanes C, E, and F) whereas the slow migrating strand is unprotected (lane B). This result implies that the fast migrating strand is the coding strand. (This assignment is being confirmed by direct sequencing of the fast migrating strand). The protection of the fast migrating strand is dependent upon the formation of DNA-RNA complexes and is not the result of secondary structure of the probe itself, since complete digestion of the probe does in fact occur in the absence of homologous yeast RNA (lane D).

Cross Hybridizing Regions

To confirm that the fast migrating coding strand is protected from S1 digestion by a transcript originating in the 5' HIS4 region as opposed to elsewhere on the genome, we probed the yeast genome for cross hybridizing sequences. Total yeast DNA was digested with either SalI or EcoRI, electrophoresed on an agarose gel, and the fragments blotted onto nitrocellulose. The ^{32}P labelled 148 bp HinfI fragment was used as the hybridization probe under conditions of low stringency. The autoradiogram in Figure 7 shows that the probe hybridizes only to the predicted 1.5 kb SalI and 3.0 kb EcoRI fragments of the HIS4 region. The absence of cross hybridizing regions from other parts of the genome argues that the fast migrating strand is protected from S1 digestion by a transcript from the 5' HIS4 region.

Primer Extension

The existence of a transcript corresponding to the 5' flanking region of HIS4 was further confirmed by primer extension experiments. The 5' $[\gamma-^{32}P]$ labelled fast migrating strand was used as a primer for reverse transcriptase in a reaction with total genomic yeast RNA (6). When the reaction was run on an 8% denaturing polyacrylamide gel, two discrete bands of increased molecular weight appeared (data not shown). The appearance of these bands is dependent upon the presence in the reaction mix of the fast migrating strand. No bands appear when the slow migrating strand is used as a primer. A more detailed analysis is in progress to define the in vivo transcriptional start site(s) of the upstream HIS4 transcript(s).

DISCUSSION

AAS Genes

We have described the isolation and characterization of 43 aas mutants. These mutations define four unlinked genes, AAS101, AAS102, AAS103, and AAS104. Complementation analysis indicates that AAS101 and AAS104 are two new
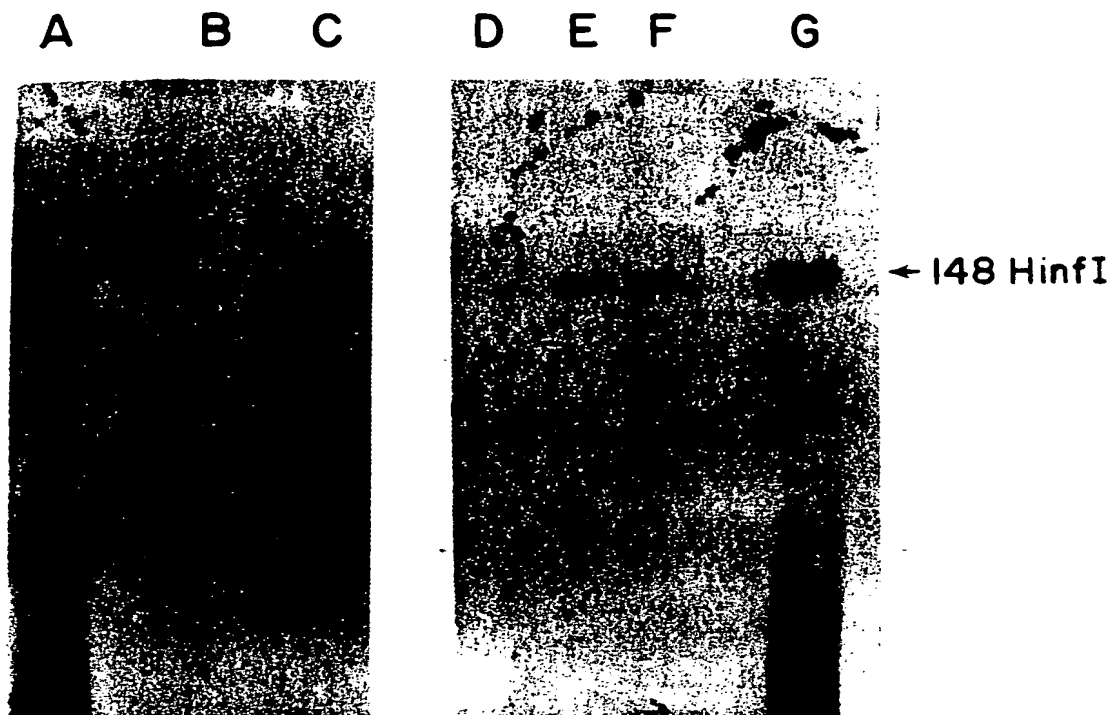
Figure 6.   Specific protection of the 148 base HinfI fast migrating
strand from S1 nuclease.

The 148 bp HinfI fragment was labelled at its 5' end with $[\gamma-{}^{32}P]$,
denatured and separated into fast and slow migrating strands on a 5%
acrylamide gel (7).   Total yeast RNA was hybridized to each of the strands,
digested with nuclease S1, and the annealed complexes resolved on an 8%
denaturing gel (1).   The control lane A contains the 148 bp HinfI fragment,
lane B contains the hybridization with the 148 base slow strand, and lane C
contains the hybridization with the 148 base fast strand.

In a separate experiment the 148 base fast migrating strand, 5' end
labelled with ${}^{32}P$, was hybridized to total yeast RNA from a culture grown in
the presence of 0.3 mM histidine (lane F) and a culture starved for histidine
with AT (lane E).   In lane D, the hybridization was carried out in the absence
of homologous RNA.   After S1 digestion, the annealed were resolved on an 8%
denaturing gel.   Lane G is an undigested control.   The exposure was at $-80°C$
with an intensifying screen.
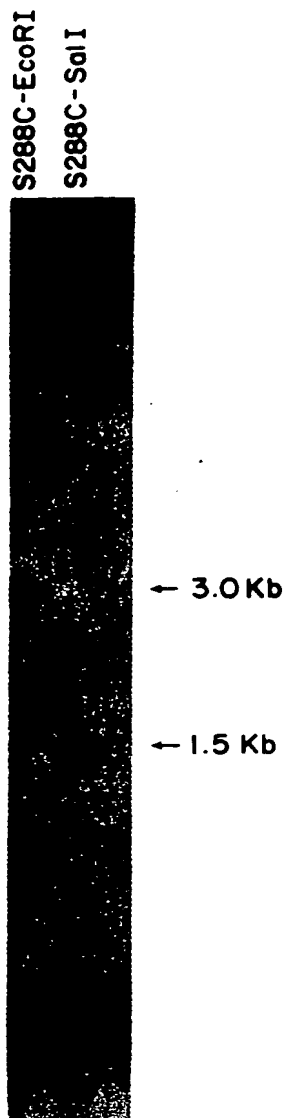
Figure 7.   Southern blot of S288C genomic DNA probed with the HinfI
fragment from the HIS4 5' flanking region. .

DNA isolated from strain S288C, cut with SalI or EcoRI, was
electrophoresed through a 1% agarose gel, and transferred to nitrocellulose
according to the method of Southern (15).   A 148 bp HinfI fragment, (-335 to
-187), was labelled at the 5' end with [γ-^{32}P] ATP by the method of Maxam and
Gilbert (7), and was used as the hybridization probe.   Hybridization was
carried out under conditions of low stringency, 55°C in 5 x SSC.   The exposure
was for 4 days at -80°C with an intensifying screen.

genes involved in general control, whereas AAS102 and AAS103 are analogous to genes identified in previous studies as (NDR2 and AAS1) and (NDR1), respectively (9, 13, 19). The aas101 and aas104 mutants may not have been isolated in earlier searches because they appear at a very low frequency: only four mutations were obtained in AAS101 and only one in AAS104, out of a total of 43 isolated in this study.

The aas101 mutants have the most severe phenotype, the aas104 has the weakest, and aas102 and aas103 are intermediary. This is apparent from the degree of their sensitivity to various amino acid analogs, their growth rates, and their HIS4C enzyme activity and mRNA levels under starvation conditions. All four AAS genes appear to be involved to varying degrees in regulating derepression of the enzymes of many amino acid biosynthetic pathways. The aas mutations seem to prevent derepression of at least the histidine, tryptophan, arginine, and methionine pathways, as evidenced by their acquired sensitivity to analogs from these four pathways. Specifically, we have assayed the HIS4C enzyme levels and have shown that under histidine starvation conditions, aas101, 102, and 103 mutants do not derepress this enzyme at all; aas104 shows only partial derepression. Northern blot analysis of these mutants for the same growth conditions indicates that the failure to derepress the HIS4C enzyme is a result of lowered HIS4 mRNA levels in the aas mutants. This result suggests that the positive regulatory role of these AAS$^+$ genes could be to promote transcription of genes coding for amino acid biosynthetic enzymes. Alternatively, the regulation could be exerted at the level of degradation of mRNA.

One aas103-complementing clone, 103R3, and two different aas101-complementing clones, 101G and 101M, were isolated. Clearly only one of the 101 clones can be the AAS101 structural gene. The 101G clone has been looked at in greater detail. Northern blot analysis of wild type S288C RNA indicates that under histidine starvation conditions, the 101G mRNA is itself greatly induced. This implies that the 101G gene is regulated in a way that directly relates to its function. If the AAS genes are positive controlling elements needed for derepression of genes under general control, an increase in AAS mRNA levels under amino acid starvation conditions is certainly logical. The Northern blot results also suggest that the regulation of the 101G gene is not mediated by the AAS102 or AAS103 gene products since the derepression of 101G mRNA occurs in strains which are mutated in these genes. (This is probably also true for the AAS104 gene, but the results are somewhat ambiguous.)

Southern blot analysis indicates that at least part of 101G sequences are present in more than one copy in the genome. Whether this reflects multiple gene copies, or the possible presence of some reiterated element, remains to be determined. Another observation is that 101M and 103R3 clones contain roughly 5 kb of inserted sequences in common. It is possible that this sequence is responsible for the observed complementation response in aas101 and aas103 mutants. Further analysis of the cloned sequences is necessary to ensure that the phenotypes which these plasmids confer are not high copy number artifacts.

5' Transcript

In this paper we have determined the existence of a transcript in the 5' regulatory region of one of the genes under general control, HIS4. The S1 nuclease experiments indicate that an in vivo transcript exists for only one of the strands of this region. Our preliminary primer extension data indicates that transcripts of this region with two distinct start sites may exist. We are currently defining precisely where these transcriptional start sites map. The upstream HIS4 transcript contains an open reading frame and has the capacity to encode a polypeptide of 66 amino acids. The fact that similarly positioned open reading frames exist upstream from the structural genes of two other amino acid biosynthetic genes, TRP5 and HIS3, strongly suggests that these putative polypeptides may have some regulatory function in the general control of amino acid biosynthesis.

Inspection of the nucleotide sequence of the 5' flanking regions reveals an intriguing property of the open reading frames. If these regions are translated, they exhibit an extreme bias for rare and infrequently used codons. These rare codons tend to be homologous to the anticodons of the minor yeast isoacceptor tRNA species. This suggests a number of regulatory mechanisms which would allow the yeast cell to monitor the charged state of the tRNAs.

Experiments are in progress to determine the involvement of the 5' transcript in HIS4 gene regulation. We are constructing inframe lacZ fusions to this region which will be used in conjunction with Northern blot analysis to correlate mRNA and protein levels with HIS4 gene expression. Another approach for determining the role of the 5' transcript, or its translation product, on HIS4 gene expression, is to construct deletions of the coding region. Typically, deletion analysis of the 5' region of yeast genes has been performed by generating deletions with an exonuclease, such as Bal31, by initiating at an upstream restriction site and deleting bases for varying distances in the direction of the promoter. Deletions generated in this manner remove all genetic homology 5' to the promoter. Moreover, the promoter is now flanked by sequences which are normally well separated. This sets up a situation which is clearly subject to artifacts. To minimize potential artifacts, we are constructing a small deletion which is totally internal to the putative coding sequences, leaving all flanking sequences intact. Analysis of a small deletion which generates a frameshift will allow us to assess the biological role of the putative translation product.

Depending upon the outcome of these experiments, a model for the role of the transcript that we have identified should become apparent. What, if any, the relationship between the AAS^+ genes and this transcript is, remains to be addressed.

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11. PROTEIN SECRETION AND ORGANELLE ASSEMBLY IN YEAST*

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Protein secretion is a major aspect of cell metabolism and provides a mechanism for assembly of internal organelles and the cell surface. The cellular functions which execute the secretory process in yeast have been identified genetically by the isolation of temperature-sensitive lethal mutants that block the secretory pathway at one of four stages. Three of these stages are defined by class A sec mutants which accumulate secretory glycoproteins inside one of three distinct organelles: endoplasmic reticulum (ER), Golgi bodies, or secretory vesicles. Glycoproteins and secretory organelles accumulate at a nonpermissive temperature (37°C) and proceed to a succeeding stage in the pathway when cells are returned to a permissive temperature (25°C) even in the absence of new protein synthesis. Another type of sec mutant (class B) fails to produce active secretory enzymes even though secretory polypeptides are synthesized. Some of the mutants in this class are blocked in the translocation of secretory polypeptides across the ER membrane.

The secretory pathway is responsible for localization of major yeast plasma membrane surface proteins. The export of at least four permease activities and six externally labeled plasma membrane proteins is blocked thermoreversibly in the sec mutants. The transport organelles, secretory vesicles in particular, may carry secreted enzymes with only a subset of plasma membrane proteins. Purified secretory vesicles do not contain chitin synthetase and vanadate-sensitive ATPase, two bonafide integral plasma membrane proteins. Another vesicle may be responsible for transport of these and other membrane proteins.

Part of the secretory pathway is also responsible for localization of vacuolar glycoproteins. Carboxypeptidase Y (CPY) is synthesized as an inactive proenzyme which is matured in the vacuole by cleavage of an 8 Kd amino-terminal propeptide. Proenzyme forms of CPY accumulate thermoreversibly in sec mutants that are blocked in movement from the ER or from the Golgi body, but not in mutants that block transport of secretory vesicles. Vacuoles isolated from sec mutant cells do not contain the proCPY produced at 37°C. These results suggest that vacuolar and secretory glycoproteins require the same cellular functions for transport from the ER and from the Golgi body. The Golgi body represents a branch point in the pathway: from this organelle vacuolar proenzymes are transported to the vacuole for proteolytic processing and secretory proteins are packaged into vesicles.

Introduction

Eukaryotic cells contain a number of membrane-bounded organelles which do not, on simple visual examination, seem to be related. Studies in the 1950's and 1960's by Palade, Porter, de Duve, and Claude led to our current view that compartmentalization provides the eukaryotic cell with a wide range of possibilities for regulation of metabolism. Recent progress on the study of the assembly of cellular organelles, in particular on the mechanism of localization of specific protein constituents, has raised the possibility of intimate connections among the organelles. The secretory process has emerged as a common theme for transport of proteins and lipids to all parts of the cell.

Protein secretion occurs in almost all cell types. Despite the wide range of activity that this implies, the stages in the secretory process are quite similar in all organisms. In eukaryotes, the sequence ER → Golgi → vesicle → cell surface is the generally accepted mode of transport for soluble and membrane proteins. Although prokaryotes clearly do not have specialized secretory organelles, polypeptide penetration across the bacterial cytoplasmic membrane occurs by processes quite analogous to those employed in protein import into the endoplasmic reticulum and into the mitochondrion.

In addition to a role in cell surface assembly, the secretory process may contribute to the assembly of the lysosome, the mitochondrion, and the nucleus. Lysosomal glycoprotein precursors are translocated into ER membranes by the same system used for secretory and plasma membrane proteins (2). Furthermore, both in histochemical and in organelle fractionation studies, lysosomal enzymes are detected in ER and Golgi cisternae (27). Mitochondrial membranes are assembled with lipids synthesized in the ER. Although many mitochondrial enzymes derive from soluble cytoplasmic precursors, the ER and mitochondrial outer membranes appear to share a number of integral proteins (29). Finally, the nuclear envelope is continuous with the ER. This is most apparent during interphase, when the nuclear envelope is reconstructed by outgrowth from the ER. Certain soluble nuclear components may also be derived from secretory organelles. The observation that chromatin-associated high mobility group proteins (HMGs) contain N-glycosidically-linked, complex oligosaccharides suggests that these proteins may gain access to the nucleus via the Golgi body (25). Clearly, secretory organelles play a major role in cell architecture and metabolism.

While a considerable amount is known about the gross features of secretory organelles, and about the structure and covalent modifications of molecules that are transported through the organelles, it has been much more difficult to define cellular functions involved in protein transport. New approaches involving genetic and biochemical techniques will be essential for appreciating the mechanism of transport. In this regard, recent advances in identifying proteins involved in the penetration of secretory and membrane polypeptides across a membrane (32,17,35), and in the discrimination of lysosomal and secretory proteins (13,14,26) provide important examples of these cellular functions.

Identifying the full range of functions required for protein transport can be achieved with a genetic approach. For this and other reasons, my laboratory has undertaken a study of the secretory process in the yeast _Saccharomyces cerevisiae_. Although much less is known about the secretory process in yeast than in mammalian cells, and in some ways there are special technical difficulties in the use of yeast as an experimental system, the potential for a combined genetic and biochemical approach will may prove a crucial advantage.

Organization of the Yeast Cell Surface

The yeast cell surface consists of at least three layers: The cell wall which contains mannoproteins and structural polysaccharides (β-1,3 and β-1,6-linked glucan), a periplasm that contains mannoproteins, and a plasma membrane. Most of the soluble secreted enzymes, such as invertase and acid phosphatase, are located in the periplasm or in the cell wall where they are accessible to low molecular weight substrates. Certain smaller non-glycosylated proteins, such as α-factor and killer toxin, are secreted through the cell wall into the culture medium.

Secretion is correlated topologically to the region of cell surface growth. Invertase and acid phosphatase are secreted into the bud portion of a growing cell which corresponds to the point of cell surface addition during most of the division cycle (31, 8). The correlation between secretion and budding is best accounted for by an exocytic mechanism of surface growth. Secretory vesicles may fuse with the inner surface of the bud and deliver mannoproteins to the periplasm and membrane precursors to the plasma membrane. The available cytologic evidence strongly supports this notion. Electron microscopic thin section and freeze fracture views show 50-100 nm vesicles which fuse with the bud plasma membrane (18). Histochemical staining of cells secreting acid phosphatase has shown enzyme-specific stain of the bud-localized vesicles, the ER, and a Golgi-like organelle (16).

Although the yeast secretory process appears to resemble the mechanism used by plant and animal cells, one striking difference is the low level of secretory organelles revealed by standard EM thin section analysis. This low level is consistent with a rapid transit time for export of invertase (20), and a low level of invertase export precursors (22). The small internal pool of secretory precursors provides a sensitive experimental system for the evaluation of mutants that block the secretory pathway and cause an accumulation of secretory enzymes and organelles.

Isolation and Characterization of Secretory Mutants

Given the possibility that the secretory process contributes generally to yeast cell surface growth, Peter Novick assumed that secretory mutants would be lethal. To get around this problem, Novick screened a collection of temperature-sensitive growth mutants for ones that failed to export active invertase and acid phosphatase at the nonpermissive temperature (37°C), but which performed normally at the permissive temperature (25°C). Mutants representing two complementation groups (sec1, sec2) were found which accumulated secretory enzymes in an intracellular pool (22). A large number of additional sec mutants have been isolated based on the observation that sec1 cells become dense at 37°C. Susan Henry showed that during inositol starvation of an auxotrophic strain, net cell surface growth stopped while cell mass increased (12). Starved cells could be resolved from normal cells on a Ludox density gradient. Similarly, sec mutant cells can be enriched from a mutagenized culture by incubation at 37°C followed by Ludox density gradient sedimentation.

The sec mutants are of two types. Class A sec mutants (192 total) are like sec1 and sec2, in that active secretory enzymes accumulate in an intracellular pool (21). Class B sec mutants (23 total) do not secrete or accumulate active secretory enzymes, yet protein synthesis continues at a near normal rate for several hours at 37°C. Complementation analysis has revealed 23 sec loci in the

A class and 4 sec loci in the B class. The distribution of mutant alleles suggests that more of both classes could be found.

Many of the class A sec mutants secrete a large fraction of the invertase that accumulates at 37°C when cells are returned to the permissive temperature. In most cases the secretion of accumulated invertase is insensitive to cycloheximide. This implies that the affected gene product is reversibly inactivated by the temperature shift. The result demonstrates that ongoing protein synthesis is not essential for post-translational transit of secretory enzymes, and thus excludes the possibility that newly-synthesized secretory protein forces the flow of the export process. Mutations in one gene (sec7) allow thermoreversible secretion only in growth medium containing a low concentration of glucose.

Perhaps the most dramatic feature of the class A sec mutants is that they accumulate or exaggerate specific secretory organelles. Mutations in ten groups produce 80-100 nm vesicles at 37°C that are distributed throughout the cytoplasm, unlike the bud-localized vesicles seen in wild-type cells. Mutations in another nine genes produce exaggerated endoplasmic reticulum. In these mutants the ER lines the inner surface of the plasma membrane and winds through the cytoplasm where multiple connections with the nuclear envelope are seen. The lumen of both the ER and the nuclear envelope is wider than the corresponding wild-type structure. Due to the high density of ribosomes in the background, it has not been possible to determine if the exaggerated ER is in the rough or smooth form. A third class of mutant, represented by two genes, produces a different organelle depending on the growth medium in which the cells are incubated at 37°C. Mutations in the sec7 gene cause the accumulation of typical Golgi-like structures when mutant cells are incubated at 37°C in medium containing 0.1% glucose. The same mutant, when incubated at 37°C in medium with 2% glucose, accumulates cup- and toroid-shaped organelles that we have called Berkeley bodies. This change in organelle morphology correlates with the effect of glucose on thermoreversible secretion mentioned earlier, and suggests that the Golgi-body structure is a more natural intermediate. In each case where reversible secretion is observed, a return to the permissive temperature allows the accumulated organelle to diminish in abundance. Histochemical staining of mutants representing each of the distinct cytologic types has shown that the secreted enzyme, acid phosphatase, is contained in the lumen of the accumulated organelle (3).

Susan Ferro-Novick has found that the class B sec mutants produce enzymatically inactive forms of invertase (5). In two of the mutants (sec53 and sec59), immunoreactive forms of invertase are produced at 37°C which appear to remain embedded in the ER membrane (6). Perhaps as a result of this aberrant accumulation, the ER in these mutants appears fragmented in contrast to the smooth, thin ER envelope seen in wild-type cells. These mutants also show greatly reduced incorporation of [^{3}H]-mannose into a total glycoprotein fraction at 37°C, although oligosaccharide synthesis is not directly affected. Reduced mannose incorporation appears to be due to a defect in translocation of nascent polypeptide chains to the lumenal surface of the ER membrane where oligosaccharides are transferred to protein. Surprisingly, protease protection experiments have indicated that a significant portion of the invertase polypeptide (11 Kd of a 60 Kd mature polypeptide length) is inserted into and perhaps across the ER membrane in mutant cells at 37°C (7). Furthermore, as with the class A sec mutants, sec53 and sec59 are thermoreversible. Upon return to 25°C, in the presence of cycloheximide, the membrane-bound form of invertase is transferred into the lumen of the ER, glycosylated, and transported to the cell surface through the normal pathway. Thus, the sec53 and sec59 gene products define functions required

for completion, but not initiation of protein penetration across the ER membrane.

Order of Events in the Pathway

A simple technique exists for the ordering of events along a linear irreversible pathway in which distinct intermediates accumulate in different mutants. In this circumstance a double mutant will accumulate the intermediate prior to the first block that is encountered. This analysis has been performed with mutants representing each of the four stages that are identified by the sec mutations. Double mutants containing sec53 or sec59 together with any of the class A sec mutants fail to accumulate active invertase (6). Thus, these class B sec mutants are epistatic to the other mutants. Among the class A sec mutants, the ER-accumulating phenotype is epistatic to the Golgi body- and vesicle-accumulating phenotypes, and a Golgi body-accumulating mutant is epistatic to all of the vesicle-blocked mutants (20). The order of events determined by this analysis is shown in Figure 1.

An independent line of evidence supports this order of events. Brent Esmon and Susan Ferro-Novick have analyzed the extent of glycosylation of invertase accumulated at 37°C in the sec strains and identified at least two stages in oligosaccharide assembly (31, 6). Very little carbohydrate is present on the membrane-bound form of invertase accumulated in sec53 and sec59, consistent with a defect in the translocation of secretory polypeptide into the ER lumen. During normal transport into the ER, invertase acquires 9-10 N-glycosidically-linked oligosaccharides which have a composition identical to the mammalian "high-mannose" oligosaccharide ($Glc_3Man_9GlcNAc_2$) (4). The three glucose residues are removed at some post-translational step in the ER because sec mutants blocked in movement from the ER have high mannose oligosaccharides with a composition of $Man_9GlcNAc_2$ (4). In addition to N-linked carbohydrate, yeast mannoproteins have O-linked mannotriose and -tetraose. The O-linked sugars appear to be formed in the ER; O-linked mannose and mannobiose accumulate in the ER-blocked mutants. Oligosaccharide maturation is completed either in transit to or within the yeast Golgi body. Mutants that block movement from the Golgi body, or the later mutants that block discharge of secretory vesicles, accumulate N-linked oligosaccharides that have a complete outer chain structure and O-linked mannotriose and -tetraose. Furthermore, incompletely glycosylated molecules that accumulate at an ER block are processed when cells are returned to the permissive temperature. The sequence of carbohydrate maturation events is summarized in Figure 2. Taken together the cytologic and biochemical results demonstrate that the yeast secretory pathway is essentially identical to the mammalian process.

Plasma Membrane Assembly

The initial characterization of the sec mutants suggested a general block in secretion and cell-surface growth at the nonpermissive temperature. In addition to an immediate halt in bud growth, export of a number of secreted proteins (invertase, acid phosphatase, L-asparaginase, α-galactosidase, α-factor, killer ... and plasma membrane permease activities ($SO_4^=$ permease, arginine permease, ... tose permease, proline-specific permease) is blocked. A more general probe ... surface assembly that was used to examine the export and turnover of macrophage plasma membrane proteins (15) has now been adapted by Novick to examine surface assembly in yeast (23). Modification of cell surface amino groups with trinitrobenzenesulfonate (TNBS) followed by precipitation with TNP antibody allows analysis of newly-exported proteins. In this procedure, wild-type and mutant
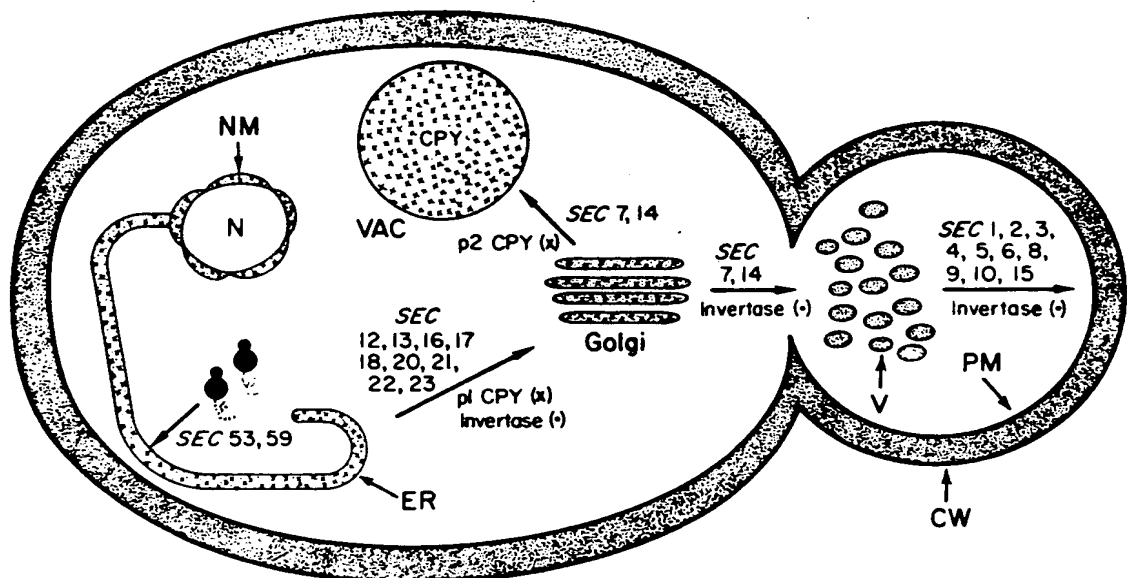
FIGURE 1.   Secretory and vacuolar protein transport pathways in yeast. N: nucleus; NM: nuclear membrane; ER: endoplasmic reticulum; SEC: wild-type genes; VAC: vacuole; V: vesicle; PM: plasma membrane; CW: cell wall, CPY: 61 Kd mature carboxypeptidase Y; p1 CPY: a 67 Kd proenzyme form of CPY; p2 CPY: 2 69 Kd proenzyme form of CPY.
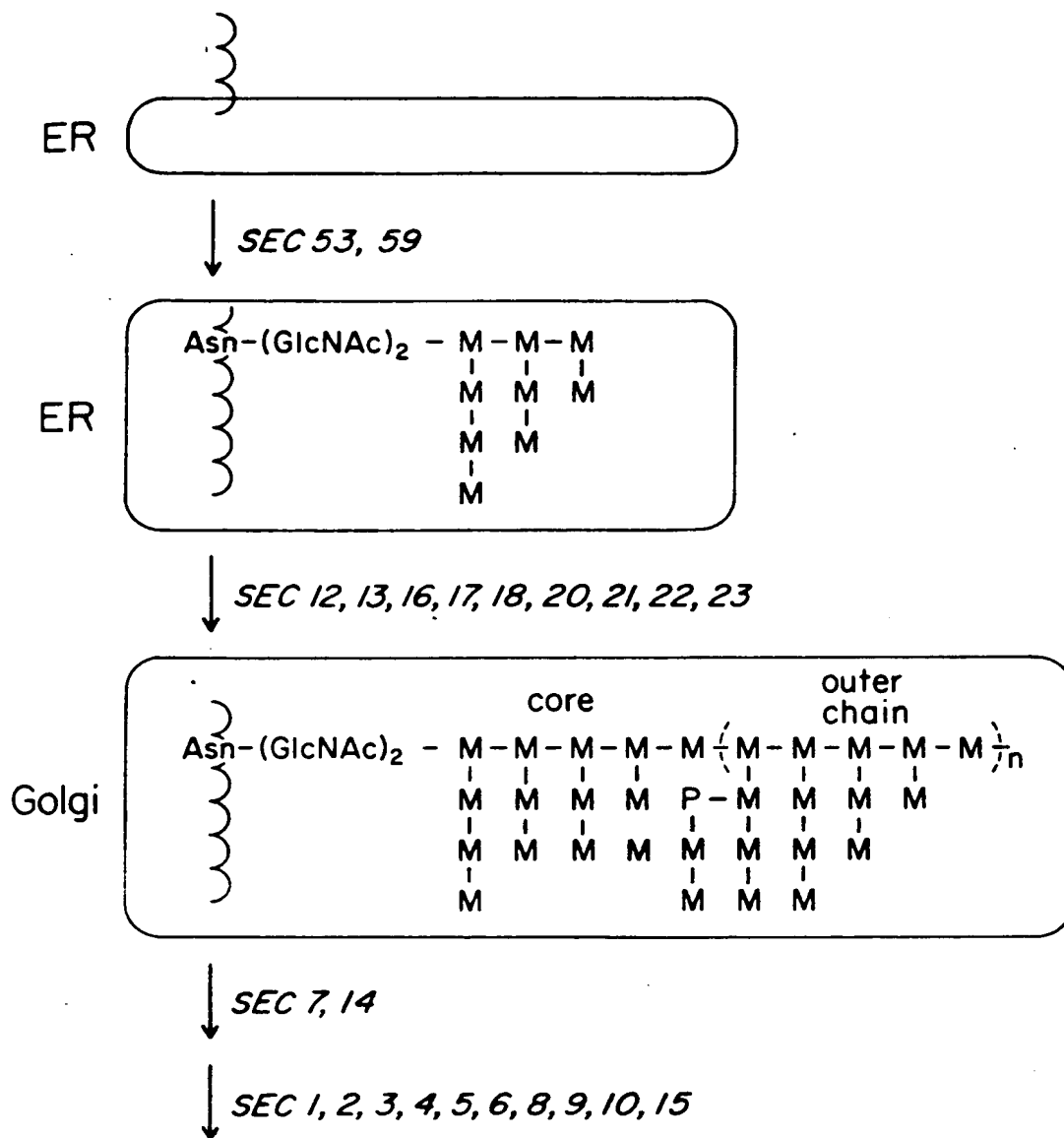
FIG. 2. Compartmentalized assembly of mannoprotein oligosaccharide. Asn, asparagine; GlcNAc, N-acetylglucosamine; M, mannose; ξ, polypeptide.

cells are labeled with protein synthesis precursors at 37°C and then tagged with TNBS at 0°C. Under these conditions, TNBS does not penetrate into the cell. Both secreted and plasma membrane surface proteins are tagged in this procedure and can be examined separately: secreted proteins are released when cells are converted to spheroplasts, and tagged membrane proteins are recovered in a sedimented fraction from lysed spheroplasts. Wild-type cells export distinct sets of membrane and secreted proteins as revealed by SDS-gel electrophoresis of TNP-antibody precipitates. The major proteins in both fractions are not exported in sec mutant cells at 37°C, but are at 24°C. Furthermore, accumulated surface proteins are exported when radiolabeled sec mutant cells are returned to 24°C. These results suggest that the secretory process is responsible for the localization of most cell surface proteins in yeast.

In order to test the possibility that secretory organelles that accumulate in the sec mutants contain secretory and plasma membrane proteins in the same structures, mutants representing each stage in the pathway have been used as a source of material in cell fractionation experiments. sec Mutant cells are induced for invertase synthesis at 37°C so that all new activity is contained within the accumulated organelle. Bill Hansen has devised a procedure for lysis of spheroplasts that retains invertase within secretory organelles. Intact organelles are monitored during membrane fractionation using latent invertase as an enzyme marker. Tina Etcheverry has developed a method for isolation of invertase-containing vesicles from the sec1 mutant. The procedure involves velocity and density sedimentation followed by electrophoresis on an agarose gel. The final fraction is free of contamination by other known cytoplasmic and membrane markers, while at least one other secretory enzyme, acid phosphatase, fractionates along with invertase. Of particular note is that two integral plasma membrane proteins, chitin synthetase and vanadate—sensitive ATPase (1,34), are synthesized in a sedimentable form in sec1 cells at 37°C but fractionate away from secretory vesicles. These two membrane enzymes, and probably others, may be transported to the cell surface in a distinct vesicle which does not behave like the invertase-containing particle. The two separable organelles may nevertheless share a requirement for sec gene products involved in transport to the cell surface.

Vacuole Assembly

The yeast vacuole contains a number of hydrolytic glycoprotein enzymes and is thus analogous to the mammalian lysosome (33). Figure 3 lists several soluble and one membrane enzyme known to be localized in the vacuole. The serine-protease carboxypeptidase Y (CPY), is a vacuolar enzyme that has been studied extensively. CPY has a single subunit of 61 Kd, 10 Kd of which is carbohydrate in the form of four N-glycosidically-linked oligosaccharides (10). Hasilik and Tanner (9) found that CPY is synthesized as a proenzyme that is converted in vivo to the mature form with a halftime of 6 min. A 69 Kd proenzyme form of CPY was found by Hemmings et al. (11) to accumulate in a mutant, pep4, which is pleiotropically defective in the maturation of a variety of vacuolar enzymes. Hemmings et al. (11) also found that a chain—terminating mutation in the CPY structural gene which results in a shortened polypeptide in a PEP4 strain, produces a fragment that is 8 Kd larger in a pep4 strain. These data suggest that proenzyme maturation requires the PEP4 gene product and is achieved by cleavage of an amino-terminal 8 Kd propeptide. Proenzyme maturation and transport does not require glycosylation. Yeast cells treated with tunicamycin, a drug which blocks the synthesis of high-mannose oligosaccharides, synthesize, transport, and proteolytically convert a nonglycosylated form of CPY (10,19).

Proenzyme proteolytic cleavage is not required for transport. Tom Stevens has shown the proCPY produced in pep4 cells is localized properly in the vacuole (30). Thus, the PEP4 gene product is required for maturation, and not for transport. If proenzyme maturation occurs in the vacuole, and if vacuolar proteins require the secretory pathway for transport, proenzyme forms will not be localized and processed at 37°C in the sec mutants.

Ferro-Novick and Hansen have shown that unglycosylated proCPY remains associated with the ER membrane in sec53 and sec59 when cells are labeled at 37°C (6). Stevens has shown that class A sec mutants that block movement from the ER or from the Golgi body accumulate proCPY in some place other than the vacuole, presumably in the accumulated organelle (30). Upon return to the permissive temperature the accumulated proenzyme forms become processed normally. Mutants that block after the Golgi step have no effect on CPY localization. These results suggest that vacuolar and secretory proteins travel together from the ER to the Golgi body where sorting may occur (Figure 1). These results rule out a secretion-recapture mechanism of localization such as has been suggested in studies on mammalian lysosomal enzyme transport (19).

A mannose-6-P determinant on N-linked oligosaccharides has been implicated in the targeting of lysosomal enzymes in human fibroblasts. Although sorting of lysosomal and secretory proteins in the Golgi body may rely on a carbohydrate structure, the ultimate source of discrimination lies in an amino acid sequence or structural feature of the targeted protein. Carbohydrate does not serve this role in yeast because at least two vacuolar proteins, CPY and an alkaline phosphatase, are synthesized and activated normally in the absence of oligosaccharide synthesis (10,24). Mutant alleles of CPY that result in misdirection of an otherwise normal proenzyme may reveal the signal responsible for normal localization.

Conclusions

The transport of cell surface macromolecules requires a large number of cellular functions. Lesions in these essential functions lead to an interruption of plasma membrane and secretory protein export at one of four stages in a linear pathway (Figure 1). Mutants blocked early in the pathway have shown that glyco-protein carbohydrate synthesis is compartmentalized: core oligosaccharides are added in the ER, and the outer chain structure is extended in the Golgi body (Figure 2). These same mutants have revealed that part of the pathway is respon-sible for localization of at least one vacuolar glycoprotein.

The sec mutants have provided a new method for tracing the pathway of protein localization in a eukaryote, and have in a limited sense defined the cellular functions required for transport. In vitro reactions that require the sec gene products and thus reflect portions of the secretory pathway will be needed to understand the mechanism of transport.

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12. MUTANTS OF YEAST OVERPRODUCING ISO-2-CYTOCHROME c*

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The following mutations cause overproduction of iso-2-cytochrome c, the minor form of cytochrome c in the yeast Saccharomyces cervisiae: CYC7-H mutations that are cis-dominant and that have extended alterations in the 5' region adjacent to the structural gene CYC7; and recessive mutations at any of the unlinked loci cyc8, cyc9, cyc10 and cyc11. Three CYC7-H mutants, CYC7-H1, CYC7-H2, and CYC7-H3 all overproduce 20-30 times the normal amount of iso-2-cytochrome c, but each have different genetic alterations that result in different abnormal sequences at different sites along the 5' region. CYC7-H1 is a reciprocal translocation with a breakpoint between the ATG initiaton codon and a XhoI site at nucleotide position -140. CYC7-H2 contains 5.5 kbp Ty element inserted at position -185. CYC7-H3 contains a deletion that extends from position -223 to approximately 5 kbp. The recessive mutants contain 3 to 7 times the normal amount of iso-2-cytochrome c although certain strains containing two of the recessive mutations overproduce approximately 15 times the normal level. Some of the recessive mutations also cause pleiotropic phenotypes not obviously related to the overproduction of iso-2-cytochrome c. The CYC7-H mutants and the four recessive mutants have higher amounts of the CYC7 transcript which approximate the overproduction of iso-2-cytochrome c. We suggest that the overproduction of iso-2-cytochrome c is due to enhancement of normal transcription either by the presence of abnormal sequences at the 5' regulatory region or by mutation of components involved in transcription of the CYC7 and possibly other genes.

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INTRODUCTION

The iso-cytochrome c system in the yeast Saccharomyces cerevisiae has been used in numerous diverse investigations, including various aspects of gene expression and regulation. As part of the studies on gene expression, we have been examining alterations that lead to increased levels of gene products. Because of a convenient selective system, mutations causing up to 30 times the normal amount of iso-2-cytochrome c have been obtained. Genetic analysis of these mutants and DNA analysis of regions encompassing the mutations revealed that overproduction is caused either by gross alterations in front of the translated region of the structural gene or by mutations at any of four loci unlinked to the structural gene. In this paper, we briefly summarize essential features of the iso-cytochrome c system and we describe these types of mutations that cause overproduction of iso-2-cytochrome c.

THE ISO-CYTOCHROME c SYSTEM

Iso-1-cytochrome c and iso-2-cytochrome c normally constitute, respectively, 95 percent and 5 percent of the total cytochrome c complement in the yeast Saccharomyces cerevisiae grown aerobically under derepressed conditions. Both iso-cytochromes c apparently carry out equivalent functions in mitochondrial oxidative phosphorylation (13) and both are more or less coordinately regulated during derepression by nonfermentable carbon sources or during induction by oxygen; however, variations in their proportions as well as in their absolute amounts occur during induction by oxygen and during the transition from a repressed state to a derepressed state (18,21). Genetic analyses of strains containing mutationally altered forms of cytochrome c have established that the primary structure of iso-1-cytochrome c is determined by the CYC1 gene (24), which is located on the right arm of chromosome X (12), and that the primary structure of iso-2-cytochrome c is determined by the CYC7 gene (4,5), which is located on the left arm of chromosome V (22). DNA sequences have been determined for the translated regions as well as the adjacent regions of both the CYC1 (26) and CYC7 (15) loci. The detailed physical information and the procedures for selecting and detecting forward mutations, reverse mutations (23) and mutations causing overproduction (4,27); make iso-cytochrome c ideally suited for investigating gene expression.

ISO-2-CYTOCHROME c OVERPRODUCERS

Mutants completely deficient in any of the mitochondrial components required for functional respiration are unable to utilize nonfermentable substrates as carbon and energy sources. Thus the double mutant, cyc1 cyc7, deficient in both iso-1-cytochrome c and iso-2-cytochrome c, is unable to grow on media containing, for example, ethanol, glycerol or lactate as the sole carbon source (4). Revertants containing as low as 1 percent of the normal amount of total cytochrome c can grow, although poorly, on glycerol or ethanol media but are still unable to grow on a special semi-synthetic lactate medium. In fact, cyc1 CYC7+ mutants lacking iso-1-cytochrome c but containing the normal 5 percent level of iso-2-cytochrome c do not grow or barely grow on lactate medium (23,28). Thus, a variety of revertants can be obtained by plating high densities of cyc1 CYC7+ cells on lactate medium. If the cyc1 allele is revertible or suppressible, revertants containing iso-1-cytochrome c can arise because of intragenic mutations or suppressor mutations. However,

if the cycl allele is not revertible or suppressible, such as in the case with
cycl deletions, then the only revertants arising on lactate medium are those
containing increased levels of iso-2-cytochrome c. The overproduction of
iso-2-cytochrome c in the various revertants ranges from barely detectable
increases to approximately 30 times the normal level. Genetic analysis
revealed that iso-2-cytochrome c overproduction can be due either to mutations
within or adjacent to the CYC7 structural gene or to mutation at any of at
least four unlinked loci. The mutations at the CYC7 locus, denoted CYC7-H
mutations, occur at very low frequencies and are associated with gross
alterations that produce dominant effects. In contrast, mutations at the
unlinked loci arise at frequencies typically observed for forward mutations
that inactivate genes. These unlinked mutations are recessive (Table 1).

Table 1.   Mutants Overproducing Iso-2-cytochrome c

Wild-type alleles	Chromosomal location	Mutant allele	Lesion	Fold increase of Iso-2
CYC7+	V left	CYC7-H1	Translocation	20-40
		CYC7-H2	Ty insertion	15-30
		CYC7-H3	Deletion	15-30
CYC8	II right	cyc8	Point	3-6
CYC9	III right	cyc9	Point	3-6
CYC10	Unknown	cyc10	Point	5-10
CYC11	Unknown	cyc11	Point	3-6

THE CYC7-H OVERPRODUCERS

A genetic and biochemical analysis of the three mutants, CYC7-H1, CYC7-H2
and CYC7-H3 indicate that all three of these mutants overproduce 20 to 30
times the normal amount of iso-2-cytochrome c, all are dominant, and all have
gross alterations adjacent to the CYC7 structural gene. The CYC7-H1 mutation
involves a reciprocal translocation, the CYC7-H2 involves the insertion of a
Ty element and the CYC7-H3 involves a deletion. The alterations adjacent to
the CYC7 locus were initially revealed from the analysis of genomic yeast DNA
probed with a CYC7+ fragment as shown in Fig. 1. EcoRI or HindIII digests of
each of the CYC7-H mutants contain one or two restriction fragments that
hybridize to the CYC7+ probe and that differ in size from each other and from
the CYC7+ fragment. The different sizes of the restruction fragments suggest
that an extensive region near the CYC7 locus is altered in each of the CYC7-H
mutants. Genetic analysis of the three CYC7-H mutants and restriction mapping
of the cloned CYC7-H2 and CYC7-H3 genes resulted in the characterization of
their corresponding aberrations.

The CYC7-H1 Mutation

An analysis of various crosses demonstrated that the CYC7-H1 mutation is a
reciprocal translocation with a breakpoint at the CYC7 locus on the left arm
of chromosome V and a breakpoint on the right arm of chromosome XVI (20). The
reciprocal translocation was identified from the pattern of spore inviability
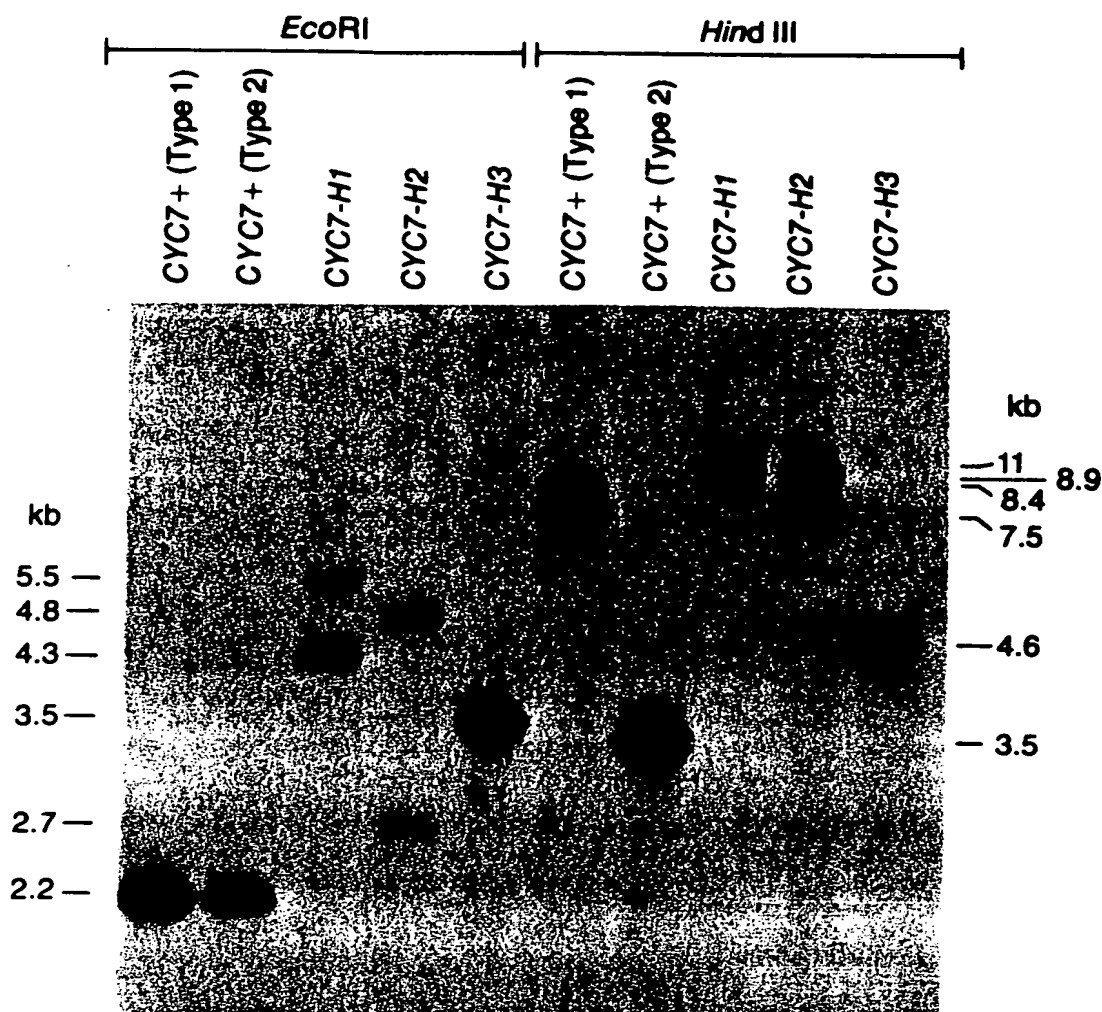
Figure 1.   Southern Blot of CYC7-H Mutants. Genomic DNA samples from CYC7+, CYC7-H1, CYC7-H2 and CYC7-H3 strains were digested with either EcoR1 or HindIII, electrophoresed in 1 percent agarose, transfered to a nitrocellulose sheet and hybridized to a nick-translated probe consisting of an EcoR1 DNA fragment containing the CYC7+ region. Type I strains lack a HindIII site 3' to the CYC7 gene, which is present in Type II strains. All of the CYC7-H mutants were derived from CYC7+ Type II strains. The autoradiogram of these digests demonstrates that fragments complementary to the CYC7+ region differ greatly in size, indicating that each of the CYC7-H1, CYC7-H2 and CYC7-H3 mutants contain different alterations within the CYC7 region. (Adapted from 19).

and abnormal linkage in heterozygous CYC7-H1/CYC7⁺ crosses. In addition, the chromosomal constitution was further characterized by the analysis of crosses that were homozygous for the translocation but heterozygous for alterations in the CYC7 structural gene. The results of the genetic analysis resulted in the genetic maps of the translocated and normal chromosomes V and XVI that are presented in Fig. 2. These genetic analyses established that one of the translocated points is at the CYC7 locus on chromosome V and that the other is proximal to mak3 on chromsome XVI (20). Dominant and recessive properties of the CYC7-H1 mutations were investigated with several mutants lacking iso-2-cytochrome c, including a UAG mutation cyc7-H1-1 corresponding to amino acid position 24. Because cyc7-H1-1/CYC7⁺ diploid strains contain approximately half of the amount of iso-2-cytochrome c found in CYC7-H1 haploid strains and because cyc7-H1-1/CYC7⁺ diploid strains contain approximately the CYC7⁺ level, the overproduction of iso-2-cytochrome c caused by the CYC7-H1 translocation is cis dominant and trans recessive.

The normal amino acid composition and peptide map of iso-2-cytochrome c from the CYC7-H1 mutant indicated that the breakpoint of the translocation is outside of the translated portion of the gene (4). Furthermore, the low frequency of CYC7-H1 recombinants among the meiotic progeny of cyc7-H1-1 x CYC7⁺ crosses is indicative of a short genetic distance between the CYC7-H1 breakpoint and structural gene (4). The abnormal size of the EcoRI and XhoI restriction fragments from the CYC7-H1 DNA shown in Figures 1 and 3 suggests that the translocation altered the EcoRI and XhoI sites that are, respectively, approximately one kbp and 140 bp in front of the translated region of the gene. These altered restriction fragments, along with finding a normal iso-2-cytochrome c, indicate that the breakpoint is between the structural gene and the XhoI site 140 base pairs in front of the AUG initiation codon. Thus it is apparent that the translocation disrupted the CYC7 regulatory region in a way that caused overproduction of iso-2-cytochrome c. The formation of an abnormal regulatory region contiguous to the CYC7 structural gene in the CYC7-H1 mutation is similar to the alteration in the other mutations CYC7-H2 and CYC7-H3, but, as described below, the regulatory region is disrupted at different sites and all three of the abnormal sequences were different from each other.

The CYC7-H2 Mutation

Initial genetic analysis established that the CYC7-H2 mutation contains a lesion at or near the CYC7 structural gene and that the lesions causing overproduction could be separated by recombination from a site in the translated portion of the gene (22). Unlike the CYC7-H1 mutation, the CYC7-H2 mutation was not associated with a gross chromosomal rearrangement that produces deficiencies in the meiotic progeny from heterozygous crosses.

The DNA alteration of the CYC7-H2 mutation was characterized by restriction endonuclease analysis of the cloned segment, by heteroduplex analysis of CYC7⁺ and CYC7-H2 segments and by DNA sequencing of the pertinent regions (8,9 and 3. Hosiba et al., unpublished results). The results, summarized in Figure 3, demonstrate that an approximately 5.5 kbp segment was inserted at a site 14 bp in front of the translated portion of the gene. The inserted segment was found to hybridize to a probe containing the central
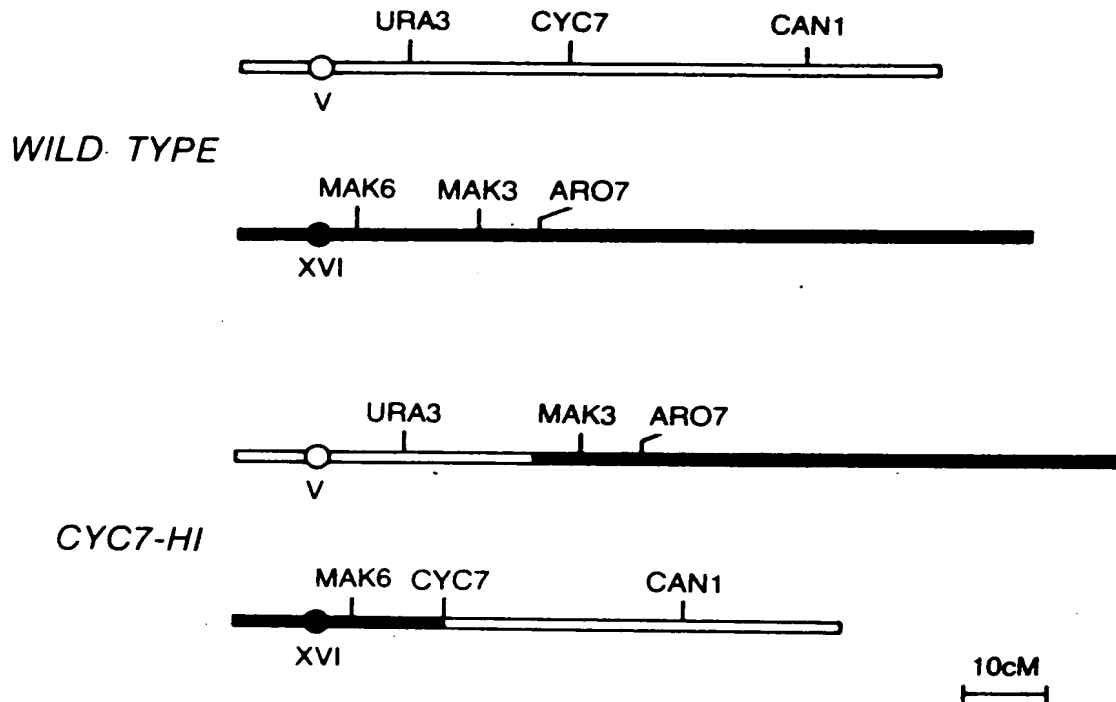
Figure 2. The CYC7-H1 Mutation. A genetic map is shown of the left arm of chromosome V and the right arm of chromosome XVI. Overproduction of iso-2-cytochrome c occurs in CYC7-H1 strains containing a reciprocal translocation between these two chromosomes (20). The breakpoint on chromosome V is adjacent to the CYC7 gene and, on chromosome XVI is between MAK6 and MAK3.
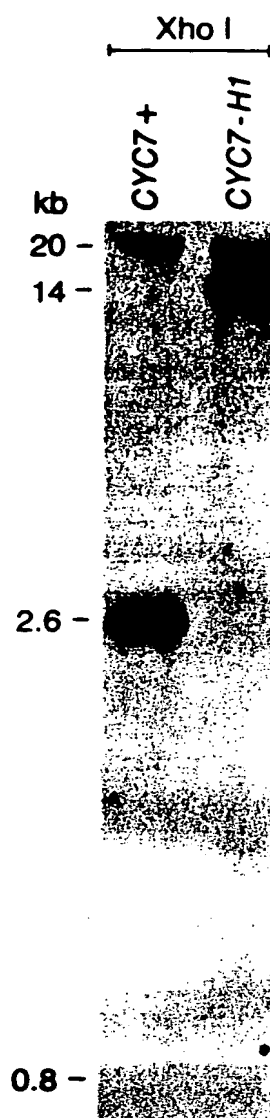
Figure 3.   Southern Blot of the CYC7-H1 Mutant.   Genomic DNA from CYC+ and CYC7-H1 DNA was digested with XhoI, electrophoresed in 1 percent agarose, transfered to a nitrocellulose sheet and hybridized to a nick translated probe consisted of an EcoRl fragment containing the CYC7+ region.   The autoradiogram shows a different XhoI restriction pattern for the CYC7-H1 and CYC+ region.

portion of a Tyl element which Cameron et al. (1) identified and characterized as a family of dispersed repetitive elements in yeast. The DNA sequence of the termini of the inserted segment were found to be closely homologous to the terminal δ sequences of known Ty elements and a 5 bp duplication was created at the site of insertion, similar to duplications created by Ty elements inserted at other sites (10,11,31).

Unlike the CYC7-H1 mutations discussed above and the CYC7-H3 mutation discussed below, the CYC7-H2 mutation has special properties that belong to a class of mutations denoted as ROAM (Regulated Overproducing Alleles responding to Mating signals) by Errede et al. (8,9).

The ROAM mutants are defined by the characteristic property that overproduction or constitutivity occurs in MATa and MATα haploid strains but less so or not at all in MATa/MATα diploid strains homozygous for the regulatory mutation. Furthermore, overproduction occurs in MATa/MATa and MATα/MATα diploid strains capable of mating as well as in specially constructed diploid strains capable of both mating and sporulating. Overproduction is decreased when haploid ROAM mutants also contain the ste7, ste11, ste12 or ste4 mutations which prevent conjugation; however, other ste mutations such as ste3, ste5 and ste13, do not affect the expression of ROAM mutations. Genetic analysis of diploid strains heterozygous for ROAM mutations and homozygous for MAT alleles indicated that the overproduction mutations are caused by cis dominant alterations adjacent to the structural genes.

In addition to CYC7-H2, the ROAM mutants include cargA$^+$O^h, which constitutively overproduces arginase, cargB$^+$O^h which constitutively overproduces ornithine transaminase, durO^h which constitutively overproduces the urea carboxylase allophanate hydrolase enzyme complex (8,9) and ADR3^c which constitutively produces slightly lower amounts of alcohol dehydrogenase II (30,31). The similar genetic properties of the cargA$^+$O^h, cargB$^+$O^h and durO^h suggest that they each contain an inserted Ty element. DNA sequencing of pertinent regions of the four mutants ADR3-2^c, ADR3-3^c, ADR3-6^c, ADR3-7^c demonstrate that they contain Ty insertions, that the points of insertion are different for each of the mutations, that the Ty elements are oriented in the same direction and that this orientation is the same as the orientation of the Ty element in the CYC7-H2 mutant. Thus, insertion of Ty elements in front of a number of unrelated genes can lead to overproduction, constitutivity or both; although the site of insertion in front of the gene may vary, it appears as if the same orientation is required and in this orientation the Ty transcript is synthesized in the opposite direction as the transcript of the affected gene (6,7).

The CYC7-H3 Mutation

Genetic and molecular analyses have demonstrated that the CYC7-H3 mutant is a deletion of about 5 kbp from the region immediately 5' to the CYC7 coding region (14). Subsequent DNA sequence analysis has shown that the deletion breakpoint proximal to the CYC7 gene is 222 bp 5' to the ATG initiation codon (Fig. 4) (Kosiba and Sherman, unpublished results). In addition to causing a cis-dominant 20-fold overproduction of iso-2-cytochrome c, the CYC7-H3 mutation was found (14) to have the following four recessive phenotypes:
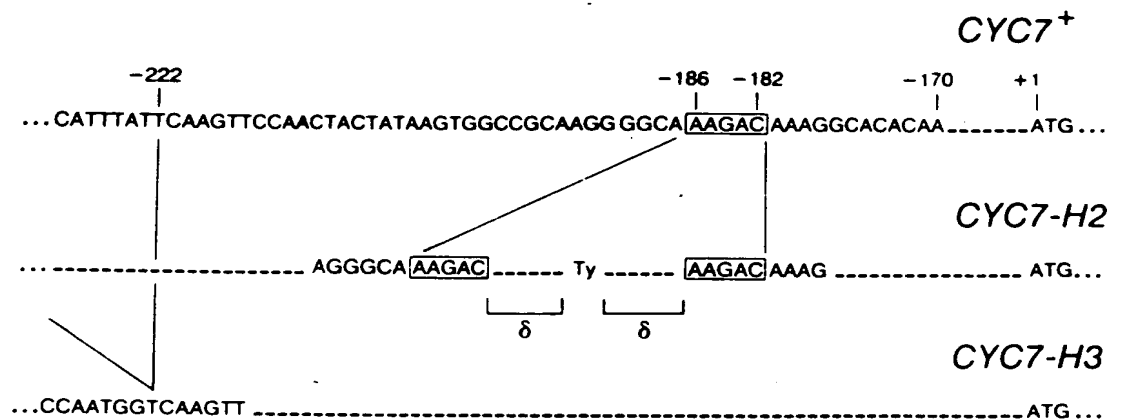
Figure 4.   The CYC7-H2 and CYC7-H3 Mutations.   The DNA sequence of the CYC7+
(15), CYC7-H2 and CYC7-H3 regions.   The CYC7-H2 sequence shows a Ty insertion
between -186 and -182 bp from the ATG initiation codon of the iso-2-cytochrome
c coding region.   This Ty insertion generates the customary 5 bp repeat at
each end of the insertion.   The CYC7-H3 sequence shows a breakpoint in the
wild type CYC7+ DNA sequence 5' to position -222.

sensitivity to ultraviolet radiation; inability to grow on hypertonic medium containing ethylene glycol or KCl; sensitivity to a breakdown product of chloramphenicol, amino nitrophenyl propanediol (ANP); and flocculent cells.

McKnight et al. (14) showed that the four recessive phenotypes segregated with the CYC7-H3 mutation in all of the 294 pedigrees examined and that they were controlled by two new genes, RAD23 and ANP1. Point mutations at the RAD23 locus failed to complement the UV sensitivity while point mutations at ANP1 failed to complement any of the other three phenotypes: ANP sensitivity, osmotic sensitivity or cell flocculence. Genetic mapping of two point mutants, rad23-1 and anp1-1, with respect to the CYC7-H2 mutation showed the gene order to be centromere-ANP1-RAD23-CYC1. From the pattern of gene conversion in this analysis the size of the ANP1-CYC7 interval was estimated at about 2.1 centiMorgans. Thus the CYC7-H3 mutation is a 5 kbp deletion extending from the ANP1 region to 222 bp in front of the CYC7 coding region which results in osmotic sensitivity and associated phenotypes, UV sensitivity and the overproduction of iso-2-cytochrome c. This overproduction of cytochrome c results from the deletion which removed 5 kbp upstream of the CYC7 gene, fusing the CYC7 gene to novel upstream DNA sequences.

RECESSIVE OVERPRODUCERS

By far the most frequent mutations causing overproduction of iso-2-cytochrome c were found to be recessive and unlinked to the CYC7 locus. Genetic complementation tests revealed that these mutations fall into four loci which have been designated CYC8, CYC9, CYC10 and CYC11 (17, Kosiba, Errede and Sherman, unpublished results). These loci may correspond to one or more of the loci CYP1, CYP2, CYP4 and CYP5 described by Verdiere and Petrochila (29) and others (3,16,28).

The cyc8 mutant

The cyc8 mutation leads to an increase in iso-2-cytochrome c production of approximately three-fold, to a lacy colony morphology and in some genetic background with abnormal cell shape (17).

The diploid cross cyc1 CYC7+ cyc8/cyc1 CYC7+ CYC8+ contains the same normal amount of iso-2-cytochrome c as the diploid cross cyc1 CYC7+ CYC8-/cyc1 CYC7+ CYC8+, thus indicating that the cyc8 mutation is recessive. Similar tests also revealed that the cyc9, cyc10 and cyc11 mutations were recessive.

The cyc8 mutation leads to an increase in iso-2-cytochrome c production of approximately three-fold, to a lacy colony morphology and abnormal cell shape in some genetic backgrounds (17).

The cyc9 Mutant

Similar to cyc8 mutants, the cyc9 mutants contain approximately three times then normal amounts of iso-2-cytochrome c. The cyc9 mutation cause a large number of apparently unrelated pleiotropic phenotypes and is, in fact, equivalent to the tup1, umr7 and flk1 mutations that were uncovered in other studies. The following pleiotropic phenotypes are associated with the cyc9

etc. mutations: the ability to utilize exogenous dTMP; a characteristic flocculent morphology; the lack of sporulation of homozygous diploids; low frequency of mating and abnormally shaped cells of a strains; the lack of detection of UV-induced mutations at the CAN1 locus, and the lack of catabolite repression of numerous enzymes including maltase, a-methyl-glucosidase invertase and succinate dehydrogenase (see (17) for original citations). However, the cyc9 mutation appears not to appreciably affect iso-1-cytochrome c or at most only slightly lower its concentration. The CYC9 locus, as well as the equivalent loci TUP1, UMR7 and FLK1 is located on the right arm of the chromsome III.

The cyc10 and cyc11 Mutants

Cyc10 and cyc11 mutants were recently uncovered in systematic searches for mutations causing overproduction of iso-2-cytochrome c (B. Errede, B. Kosiba, and F. Sherman, unpublished results). The examination of cytochrome c in diploid crosses established that the cyc10 and cyc11 mutatios are recessive and that they complement each other as well as the cyc8 and cyc9 mutation. In addition, as shown in Table 2, the cyc10 and cyc11 mutations are unlinked to each other and to the cyc8 and cyc9 mutations. The cyc10 and cyc11 mutants overproduce, respectively, approximately 7 and 3 times the normal amount of iso-2-cytochrome c, similar to the overproduction of observed in cyc8 and cyc9 mutants. However, unlike the cyc8 and cyc9 mutations, the cyc10 and cyc11 mutations do not produce any additional effects such as aberrant colony morphology.

Table 2.   Independent Segregation of cyc10 and cyc11 with Each Other and with cyc8 and cyc9

	PD	NPD	T		PD	NPD	T
cyc10-cyc8	2	2	10	cyc11-cyc8	2	2	5
cyc10-cyc9	1	2	4	cyc11-cyc9	1	1	9
cyc10-cyc11	0	3	11				

INTERACTION OF OVERPRODUCERS

Haploid strains containing various pairwise combinations of the cyc8, cyc9, cyc10 and cyc11 mutations produced higher levels of iso-2-cytochrome c than haploid strains containing only one of the mutations (Table 3). The overproduction of iso-2-cytochrome c appears approximately additive although combinations with cyc10 produce unexpectedly larger amounts of iso-2-cytochrome c. In contrast, previous results with the CYC7-H2 mutation indicated that the CYC7-H2 overproduction was not compounded with the cyc8 or

cyc9 overproduction; cyc1 CYC7-H2 cyc8 and cyc1 CYC7-H2 cyc9 segregants did not appear to contain higher levels of iso-2-cytochrome c than the cyc1 CYC7-H2 segregants (17).

Table 3.   Overproduction of Iso-2-cytochrome c in Haploids
Containing Combinations of Recessive Mutations

Single Mutations		Double Mutations		
Genotype	Fold overproduction	Genotype		Fold overproduction
cyc8	3	cyc8	cyc9	6
cyc9	3	cyc8	cyc10	15
cyc10	7	cyc8	cyc11	6
cyc11	3	cyc9	cyc10	15
		cyc9	cyc11	6
		cyc10	cyc11	15

OVERPRODUCTION OF THE CYC7 TRANSCRIPT

Overproduction of iso-2-cytochrome c in the three CYC7-H mutants and the four cyc8, cyc9, cyc10 and cyc11 recessive mutants approximately parallels overproduction of CYC7 transcription. Total cellular RNA, extracted from each of the overproducers, was separated on a denaturing agarose gel, transferred to a nitrocellulose membrane and hybridized with a specific CYC7 probe. The results, shown in Figure 5, demonstrate that little CYC7 mRNA is produced by the CYC7+ strain compared to the strains overproducing iso-2-cytochrome c. Although precise quantitative determinations were not made, the level of CYC7 transcription was higher in the three CYC7-H mutants than the four recessive mutants, suggesting that the amount of the iso-2-cytochrome c protein corresponds to the amount of the CYC7 transcript.

CONCLUSION

Mutations overproducing iso-2-cytochrome c consist of either gross structural changes upstream of the coding region or extragenic mutations at several unlinked loci. The former class of mutations includes a 5 kbp deletion, a 5.6 kbp insertion and a reciprocal translocation between chromosomes V and XVI. Each of these mutations introduces entirely new DNA sequences within the first 230 bp upstream of the iso-2-cytochrome c ATG translation start site. These abnormal DNA sequences may function directly, by providing the basic DNA sequence necessary to bind RNA polymerase and initiate transcription at abnormally high rates. Alternatively, these sequences may function indirectly by enhancing the utilization of pre-existing signals by some unknown means such as altering the chromatin structure.
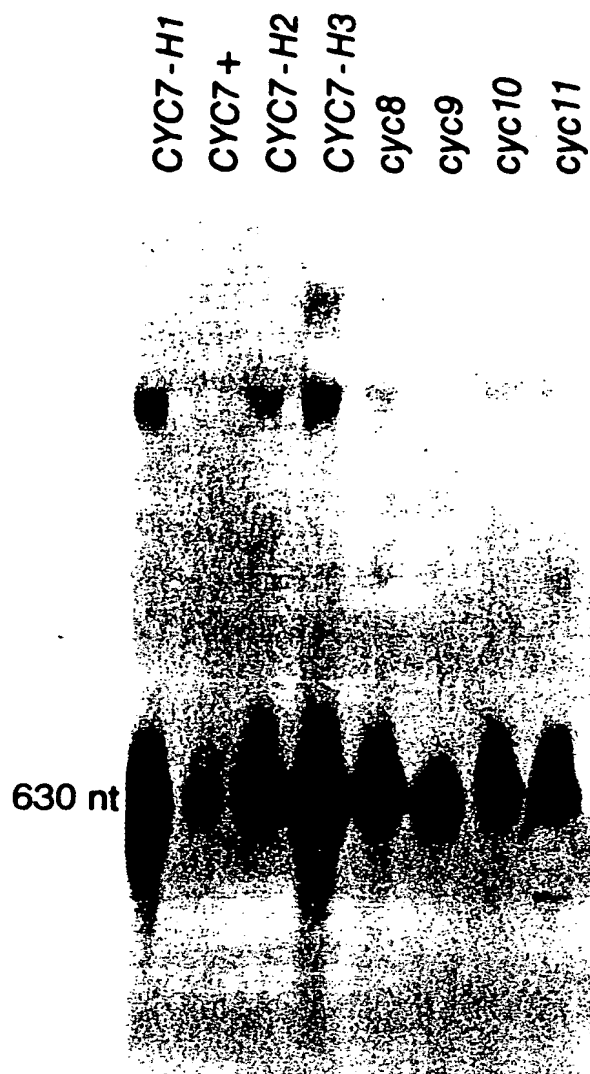
Figure 5.   CYC7 Specific mRNA in Iso-2-cytochrome c Overproducer Mutants.
Total cellular RNA was extracted from CYC7+ and iso-2-cytochrome c
overproducer strains. Approximately 20 μg of each sample was denatured, run
in 1.8 percent agarose, 50 percent formamide, transfered to a nitrocellulose
sheet and hybridized to single strainded radiolabeled probe consisting of a
Sau3AI/HpaII fragment containing most of the CYC7 cistron.   The autoradiogram
shows a low level of 630 nt CYC7-specific message from CYC7+ cells, moderately
increased levels of CYC7-specific message from cyc8, cyc9, cyc10 and cyc11 RNA
and greatly increased levels of CYC7-specific message from CYC7-H1, CYC7-H2
and CYC7-H3 cells.

The CYC7-H1 mutation contains new DNA sequences less than 140 bp from the CYC7 ATG initiation codon and produces a transcript which appears to be slightly shorter than that of the wild type CYC7⁺ gene. Thus the overproduction of iso-2-cytochrome c seen in CYC7-H1 mutants may result from an abnormal 5' region determining the rate and origin of CYC7 transcription. However, the CYC7-H2 and CYC7-H3 transcripts both appear to be the same size as the CYC7⁺ transcript. Although the mechanisms of overproduction in CYC7-H2 and CYC7-H3 are unknown, a common mode of action can be suggested by considering the properties of CYC7-H2 and other ROAM mutations. In the case of the CYC7-H2 and four ADR3ᶜ mutants, the transcripts of the affected gene and the adjacent Ty element are synthesized divergently. The size of the CYC7-H2 transcript (Fig. 5) and the 5' ends of seven ADR3ᶜ transcripts (31) indicate that the mRNAs in ROAM mutants are initiating at the normal site. In addition, the amount of Ty RNA is dependent on the mating type system, there being 20 times more in MATa and MATα haploid cells than in MATa/MATα cells (7). Thus it appears that transcription away from the 5' end of certain genes causes overproduction and constitutivity. Also, the insertion of just any DNA sequence does not cause equivalent effects; there is a lack of constitutive expression of alcohol dehydrogenase II when an insert of approximately 350 bp solo δ is at the same site as the inserted Ty element in ADR3ᶜ mutants (2). In fact, the insertion of a solo δ sequence at various sites causes diminution and complete deficiency of the gene product. These results, together with the CYC7-H2 and other ROAM mutations, lead us to suggest that the overproduction in the CYC7-H3 mutant may also be due to transcription away from the CYC7 gene on the abnormal DNA sequence. The hypothetical transcript should not, however, be diminished in MATa/MATα cells. A more complete analysis of the abnormal DNA region in the CYC7-H3 mutant and an analysis of the corresponding transcripts may reveal whether or not there is a similar mode of action between overproduction in the CYC7-H2 and CYC7-H3 mutants.

The second class of mutations includes alterations at four unlinked loci which cause increases of 3 to 7 fold in iso-2-cytochrome c production. While it is possible that one or more of these mutations is within the gene of a specific regulator of iso-2-cytochrome c production, the pleiotropic nature of two of them (cyc8 and cyc9) suggests that this may not be the case. In fact, the extreme pleiotropy of the cyc9 mutant suggests that it plays a fairly nonspecific, though important role in determining levels of gene transcription. It is tempting to speculate that the recessive mutations destroy or modify components required for normal transcription or for chromatin integrity which indirectly modify transcription.

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13. EXPRESSION, PROCESSING, AND SECRETION OF HETEROLOGOUS GENE PRODUCTS BY YEAST

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A DNA sequence, capable of transcription starting specificity in Saccharomyces cerevisiae (yeast), has been isolated from the 5'-flanking sequence of the yeast 3-phosphoglycerate kinase (PGK) gene by introduction of specific restriction sites between this flanking sequence and the ATG-translational start. This portable PGK promoter fragment has been used to express the genes for both mature and pre-forms (secretion signal sequence present) of human interferons. All of the expressed mature forms remain in the yeast cell, while the pre-forms are found outside as well as inside the cell with some of the interferon activity also present in the cell wall. The proportions of interferon secretion vary somewhat depending on which interferon gene is expressed and at what stage of cell growth the interferon is assayed. One gene product, expressed from the leukocyte A (LeIF A) gene containing a hybrid secretion signal derived from LeIF D and LeIF A pre-sequences, has been purified from both outside and inside the cell. The interferon found free in the medium is mostly properly processed LeIF A by amino-terminal analysis, while another form containing 3 additional amino acids of pre-sequence is also present. The interferon purified from the cell is also all processed; however, three forms are present with proportions that are different from extracellular forms.

INTRODUCTION

We have previously described a plasmid which is capable of expression in yeast of the mammalian gene product, human leukocyte interferon (1,22). The expression of this heterologous gene in yeast was obtained by the proper insertion of the structural gene between two yeast derived sequences in a plasmid capable of replication and selection in both yeast and E. coli. The 5'-flanking DNA sequence (promoter fragment) used to obtain transcription was derived from the 5'-flanking sequence of the yeast alcohol dehydrogenase I (ADH1) gene and the 3'-flanking sequence (transcription terminator) was the 3'-end of the TRP1 gene (31,33) from yeast. The structural gene was a modified cDNA made by replacing the 23-amino acid signal peptide sequence with an ATG-translational initiation codon preceded by an EcoRI linker (R. Gray and D.G., unpublished results).

The ADH1 promoter fragments used for initiation of transcription were isolated by Bal31 exonuclease digestions from a restriction site within the ADH1 structural gene through the ATG translation start, followed by the addition of an EcoRI linker at the 3'-ends of fragments of various lengths.

We describe here the construction of a new yeast promoter fragment from another highly expressed yeast gene; that is the gene for 3-phosphoglycerate kinase. The gene that codes for this enzyme in Saccharomyces cerevisiae has been isolated, described, and characterized (21). Furthermore, evidence suggests that there is a single copy of this gene per haploid cell that is responsible for the high expression (20). We have obtained additional evidence that the gene isolated above is that for 3-phosphoglycerate kinase; in that the DNA sequence shows an amino acid sequence homologous with human (23) and horse (3) PGKs. We here describe a portion of the DNA sequence from this gene and how this sequence information was used to add specific restriction sites, so that this promoter fragment can be used as a portable restriction fragment for the construction of plasmids to express heterologous gene products in yeast.

Using the PGK promoter fragment in a $2\mu m$ origin-based (7) plasmid, we have expressed the cDNA genes for both mature and pre-interferons. Pre-interferon genes code for mature interferons as well as the natural human amino acid sequences which signal secretion of these proteins from the human cell and which are cleaved during this process to give mature interferons (13). Mature interferon genes have been constructed in vitro from cDNA sequences by removal of the signal coding DNA sequence and replacement of this sequence with an ATG translational start preceded by a restriction site (15). Expressing such a modified gene in E. coli (15) or yeast (22) results in the formation of mature interferon activity within the cell without secretion. However, we now report that when the human secretion signal sequences are not removed but expressed using the PGK promoter, interferons are secreted into the yeast cell wall and into the medium. Although this process varies in efficiency from one interferon gene to another, it is a true secretion process; that is, it is not the result of partial cell lysis. One of the pre-interferon gene products (LeIF A activity) has been purified from both cell extract and medium. The interferon protein from the medium is composed of two forms. The major form (60-70 percent) is properly processed mature LeIF A, identical to the mature interferon that results after secretion from human cells. This demonstrates that the low eukaryote yeast is capable of recognizing and processing the secretion signal of the highest eukaryotic organism. Another form (30-40 percent), having 3 extra amino acids of pre-sequence at the amino terminus suggests that yeast does not process the protein with perfect fidelity. The nature of the interferon remaining inside the cell is even more complex since three forms are present. This variation in processing may be related to the efficiency of secretion to the outside of the cell.

MATERIALS AND METHODS

Strains and Media

E. coli K-12 strain 294 (2) was used for all bacterial transformations. Yeast strains 20B-12 (a trp1 pep4-3) (24) and GM3C-2 (a, leu2-3 leu2-112 trp1-1 his4-519 cyc1-1 cyp3-1) (12) were used.

Luria broth was as described by Miller (27) with the addition of 20 µg/ml ampicillin. Yeast were grown on the following media: YEPD contained 1 percent yeast extract, 2 percent peptone and 2 percent glucose. YNB+CAA (used for Trp+ selection) contained 6.7 grams of yeast nitrogen base

(without amino acids) (YNB) (Difco), 10 mg of adenine, 10 mg of uracil, 5 grams Difco casamino acids (CAA), and 20 grams glucose per liter. Solid medium contained 3 percent agar.

Plasmid DNA Preparation, Transformations, and Stabilities

Purification of covalently closed circular plasmid DNAs from E. coli (9) and transformation of E. coli (8) were done in accordance with previously described procedures. E. coli miniscreens were as described by Birmboim and Doly (5). Transformation of yeast was done essentially as previously described (19). Stability of plasmids in yeast was determined by replica plating colonies from YEPD (nonselective) to YNB+CAA (selective) media.

Extract Preparation and Interferon Assays

Extracts and media from yeast were assayed for interferon by comparison with interferon standards using the cytopathic effect (CPE) inhibition assay (30). Media were assayed directly after cell removal while yeast extracts were prepared as follows: Cultures were grown in YNB+CAA and 10 ml aliquots of cells were collected by centrifugation then resuspended in 3 ml of 1.2 M sorbitol, 10 mM KH_2PO_4 (pH 6.8) and 1 percent zymolyase 60,000 followed by incubation at 30°C for 30 min (to 90 percent spheroplasting). Spheroplasts were pelleted at 3000 xg for 10 min., then resuspended in 150 µl of 7M guanidine hydrochloride (GHCl). Extracts were diluted in PBS/BSA buffer (20 mM NaH_2PO_4 (pH 7.4), 150 mM NaCl, and 0.5 percent BSA). Alternatively, 10 ml of cells at the same A_{660} were pelleted and resuspended in 0.4 ml of 7M GHCl in an Eppendorf (1.5 ml) tube containing about 0.4 ml of glass beads (0.45 to 0.5 mm, B. Braun Melsurgen AG). These tubes were vortexed twice for 2 min at the highest vortex setting, keeping on ice in between. The extracts were centrifuged 0.5 min in an Eppendorf centrifuge and diluted in PBS/BSA buffer as above. Bioassays were performed with MDBK cells (30) for LeIF A, LeIF D, and the pre-forms, but with HeLa cells (30) for IFN-γ and preIFN-γ.

Purification of (pre D/A) LeIF A from the Medium and Cell Extract

Yeast strain YEp1PT-preLeIF A53t/20B-12 was grown at 30°C to A_{660} of 4. At this time the 5 liter culture was harvested by centrifugation. Ten milliliter aliquots were withdrawn periodically during the fermentation to measure optical density, interferon production, and secretion. The medium and cells were assayed as described above.

The medium was concentrated and diafiltered in an Amicon thin channel apparatus or a 2.5 liter stirred cell. The retentate was further purified by ion exchange chromatography on CM-52 followed by immunoaffinity column chromatography as described for LeIF A (29). The peak of activity was lyophilized and the residue redissolved in 0.1 percent trifluoroacetic acid (TFA), pH 2.5. The sample was then further purified by high pressure liquid chromatography (HPLC) on a Synchropack RP-P column. The column was eluted at a flow rate of 1 ml per minute with a linear gradient of 0 to 100 percent acetonitrile, 0.1 percent TFA, pH 2.5 in 60 minutes. The protein in the peak fractions containing interferon activity was then sequenced at the amino terminal end.

The cells harvested above for medium isolation were disrupted in a Bead
Beater (Biospec Products). The lysate was centrifuged, the pellet washed,
and the supernatants combined. The supernatants were dialyzed and the
interferon purified as described above for medium material. A portion of
the interferon was further purified by HPLC with a linear gradient of 0 to
100 percent acetonitrile. The protein from fractions containing interferon
activity was then sequenced at the amino terminal end.

Protein Sequence Analysis

Sequence analysis was based on the Edman degradation (10). The sample
was introduced into the cup of a Beckman 890B spinning cup sequencer.
Polybrene™ (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was
used as a carrier in the cup (32). The sequencer was modified with a cold
trap and some program changes to reduce background peaks. The reagents were
Beckman's sequence grade 0.1 molar Quadrol buffer, phenylisothiocyanate, and
heptafluorabutyric acid.

A modification also included automatic conversion of the
2-anilino-5-thiazolinone derivatives as they were extracted from the
sequencer. The 1-chlorobutane was collected in a Pierce Reacti-Vial™ and
dried under nitrogen. Then 25 percent TFA in water was added to the
2-anilino-5-thiazolinone and heated to $70^\circ C$ for 10 min to convert it into
the 3-phenyl-2-thiohydantoin (PTH derivative) (35). The PTH-amino-acid
residue was then automatically dissolved in 50 percent acetonitrile and
water and injected into a reverse-phase high-pressure liquid chromatograph.
Each PTH-amino acid was then identified by comparison to the retention times
of a standard mixture of PTH-amino acids.

RESULTS

Identification of PGK 5'-flanking DNA Sequence

Fig. 1 illustrates a partial restriction map of a cloned 3.1 kbp HindIII
fragment from chromosome III of yeast, which contains the gene for yeast
3-phospnoglycerate kinase (20,21). We have determined the exact location of
the structural gene by DNA sequencing from the PvuI site to the HindIII
site. The relevant portion of the sequence is shown in Fig. 1 as an insert
between the Sau3A through the ATG translational start and 8 amino acids of
additional coding sequence. This is thought to be the start of the
structural gene due to the length of the open reading frame (consistent with
polypeptide size), the DNA sequence 5' to the ATG, and extensive homology of
protein sequence between yeast PGK and human (23) or horse (3) sequence.

Construction of a PGK Promotor Fragment

Having identified the start of the structural gene, a design was
required for the isolation of a promoter fragment (5'-flanking sequence of
the PGK gene) on a specific restriction fragment for expression of fused
heterologous genes (portable promoter fragment). This was done for the
alcohol dehydrogenase I (ADH1) gene by Bal31 digestion through the ATG into
the 5'-flanking sequence followed by the addition of an EcoRI linker (22).
Since deletion of up to 32 base pairs of 5'-flanking sequence adjacent to
the ATG had no gross effects on the expression of a heterologous gene

THE INSERTION OF AN <u>Eco</u>RI SITE IN THE 5' FLANKING DNA
OF THE 3-PHOSPHOGLYCERATE GENE OF YEAST

DNA SEQUENCE OF THE 5' END OF THE
YEAST 3-PHOSPHOGLYCERATE KINASE
STRUCTURAL GENE AND FLANKING DNA
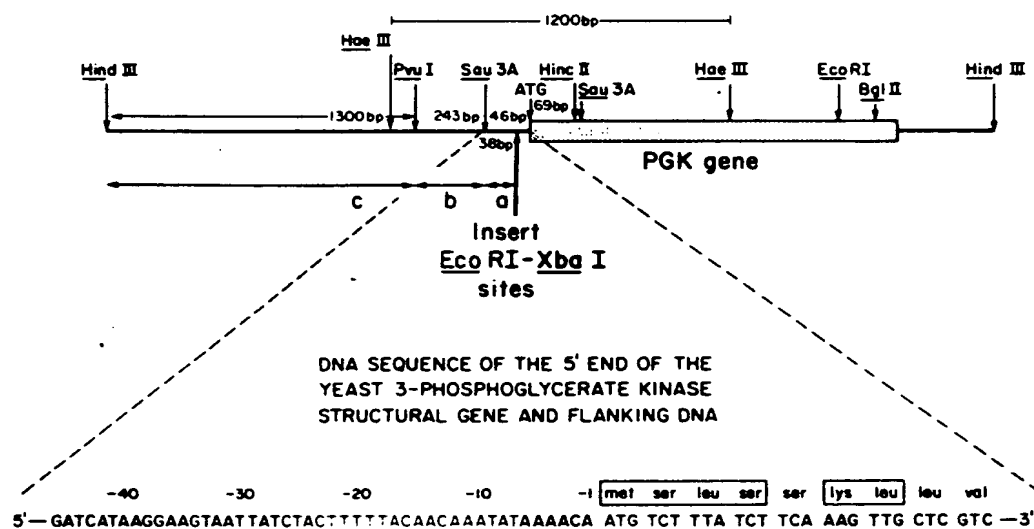
Fig. 1. Partial restriction and sequence map of the PGK gene. The PGK structural gene is shown as a bar region in the partial restriction map of a 3.1 kbp HindIII fragment. Note that distances are not drawn to scale; however, distances of fragments relevant to the discussed constructions are designated. The inserted 5' to 3' sequence (left to right) is from the ____ site through 3 amino acids of coding sequence. The underlined sequence was used to make a complementary oligonucleotide for the primer repair reaction. Boxed in amino acids are identical to amino acids in the amino-terminal end of horse and human PGKs.

(leukocyte interferon D), Bal31 digestions were not used for construction of the PGK promoter fragment.

Instead, we used the primer repair technique described by Goeddel et al. (14). An oligonucleotide (12 nucleotides long) was synthesized which is complementary to the -9 through -20 nucleotide sequence of the PGK strand shown in the insert of Fig. 1. When such an oligonucleotide is hybridized with denatured DNA from this region of the PGK gene in the presence of Klenow DNA polymerase I, polymerization occurs 5' -> 3' through the upstream Sau3A site while the 3' -> 5' exonuclease degrades phosphodiester bonds from the 3' end of the fragment until the double stranded region is reached. By cutting tne repair product with Sau3A a small region of the PGK promoter fragment can be isolated as a Sau3A to blunt end DNA (-46 to -9) fragment. The loss of -8 through -1 of the 5'-flanking sequence was thought not to be a problem due to the ADH1 promoter fragment results already discussed.

The PGK fragment which contains a blunt end and a Sau3A sticky end (sequence "a" in Fig. 1) can then be ligated into a plasmid vector containing a BamHI sticky end and a filled-in XbaI blunt end. This allows a portion of the 5'-flanking sequence of the PGK gene to then be isolated from this vector as a Sau3A to XbaI restriction fragment. However, since this fragment is small and since transcriptional starting efficiency and control of yeast genes is sometimes affected by yeast DNA far upstream from the ATG translational start (12,17), the plan to make a PGK promoter fragment required the reconstruction of 5'-flanking sequence upstream from the Sau3A site to the HindIII site (thus sequences "b" and "c" in Fig. 1 were added back to sequence "a"). During this reconstruction an EcoRI site was also added adjacent to the XbaI site so that the 1600 bp promoter fragment can be isolated as a HindIII-to-XbaI or as a HindIII-to-EcoRI fragment.

A Yeast Plasmid for Expression of Heterologous Genes

A plasmid containing the PGK promoter fragment and other necessary components is shown in Fig. 2. This plasmid contains a portion of pBR322 (6) with the ampicillin resistance (Ap^R) gene and the E. coli origin of replication for selection and stable growth in E. coli. The plasmid also contains the TRP1 gene on an EcoRI to PstI fragment which originates from chromosome IV of yeast (31,33). This gene allows for selection and maintenance of the plasmid in trp1 yeast. The plasmid also contains a yeast origin of replication on a 2.0 kbp fragment from endogenous yeast 2μm plasmid DNA (7). This origin allows the DNA to replicate autonomously in yeast and be maintained as a plasmid.

Furthermore, the plasmid system contains the yeast PGK promoter fragment which originates transcription near the only EcoRI site in the plasmid (the other EcoRI site was removed by standard procedures). A HindIII/BglII fragment from the yeast TRP1 gene region (31,33) of chromosome IV was used as a convertor of HindIII to BglII for ligation with the BamHI site of pBR322. The 2.0 kbp fragment from 2μ DNA contains the transcription termination/polyadenylation signals which are normally the signals for tne "Able" gene in 2μm plasmid (18). Such a region appears to be essential for such a yeast expression system (data not shown) as well as for a natural yeast gene (36). Gene inserts as EcoRI fragments in the proper orientation can thus be expressed as protein when the vector is put into yeast. Many

YEAST EXPRESSION PLASMID
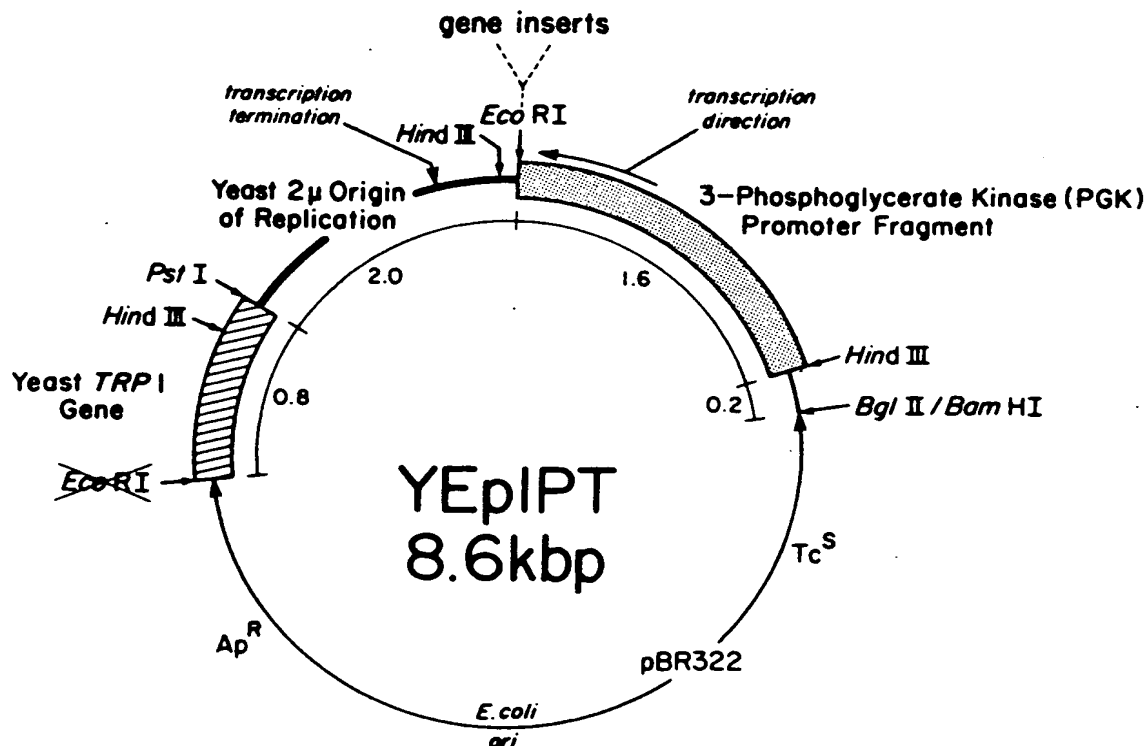
Fig. 2. Yeast expression plasmid. The partial restriction map of YEp1PT is shown with components designated that are required for transcription and translation of a heterologous gene inserted at the single EcoRI site.

heterologous genes have been expressed in this system. Some examples besides the interferon genes mentioned here are human serum albumin (26) and hepatitis surface antigen (34).

Interferon Genes

Fig. 3 illustrates some EcoRI fragment constructions containing interferon cDNAs that were used in the YEp1PT expression plasmid. The details of these constructions will only briefly be discussed here. The modifications involved in the construction of mature LeIF D (22), LeIF A (15), and IFN-γ (16) have already been published. Mature LeIF A required a final modification which we refer to as a converter. This 245 bp DNA fragment was obtained from yeast 2um plasmid DNA (18) and due to its location was thought to be devoid of transcripion terminators. However, recent data have shown that there is a terminator on this fragment which functions as well as the "Able" terminator. Pre-interferon 5'-ends were converted to EcoRI by the primer repair technique (14) or by conversion of a convenient restriction sites.

Expression and Secretion of Interferons

The genes shown in Fig. 3 were inserted into YEp1PT (Fig. 2), and yeast were transformed with these plasmids. Transformants were assayed for interferon using the cytophathic effect bioassay (30). Interferon assays were done on three distinct compartmental locations in the yeast culture. The results of such assays are shown in Table 1.

INTERFERON GENES USED IN YEAST EXPRESSION PLASMID
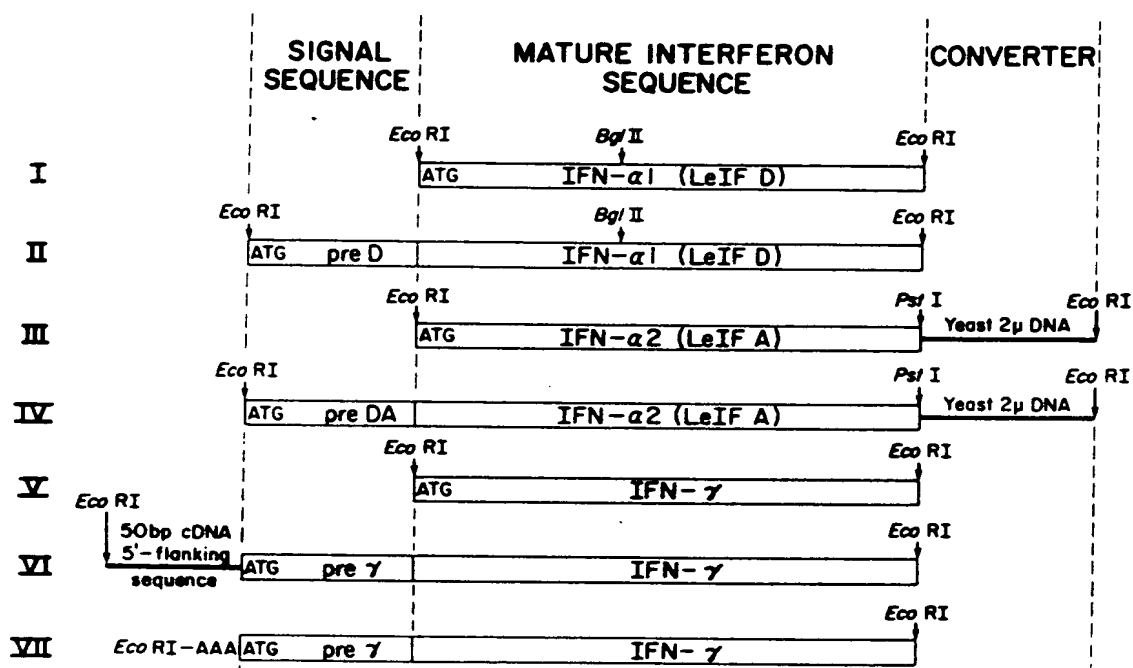
Fig. 3.   Modifications of cDNAs for insertion into the EcoRI site of YEp1PT.

Table 1. Interferon Expression Levels

Gene Construction No.	YEp1PT plasmid containing these EcoRI fragments	Yeast	Inside cell U/1/Abs660-1 [a]	Pct. cell protein [b]	Released after cell wall removal (U/1/Abs660) [c]	Pct. cell protein	Outside cell (media) U/1/Abs660	Pct. cell protein	Final Abs660 [d]	Pct. of activity secreted [e]
I	LeIF D	GM3C-2	130×10^6	1.0	0	0	0	0	1.0	0
II	pre LeIF D	GM3C-2	27×10^6	0.3	0.4×10^6	.004	0.8×10^6	.008	1.4	4
III	LeIF A	20B-12	130×10^6	1.0	0	0	0.5×10^6	.005	1.0	5
IV	(pre D/A) LeIF A	20B-12	19×10^6	0.2	0.5×10^6	.005	0.5×10^6	.005	1.0	5
IV	(pre D/A) LeIF A	20B-12	25×10^6	0.1	N.D.	—	2×10^6	.007	3-4	8
IV	(pre D/A) LeIF A	GM3C-2	28×10^6	0.3	0.3×10^6	.003	0.5×10^6	.005	1.3	3
V	IFN-γ	20B-12	0.6×10^6	N.D.	N.D.	—	0	0	1.0	0
VI	pre IFN-γ + cDNA 5' flanking sequence	20B-12	0.2×10^6	N.D.	N.D.	—	$.03\times10^6$	N.D.	1.2	15
VI	pre IFN-γ + cDNA 5' flanking sequence	GM3C-2	0.38×10^6	N.D.	N.D.	—	$.06\times10^6$	N.D.	0.93	16
VII	pre IFN-γ	20B-12	0.9×10^6	N.D.	N.D.	—	$.19\times10^6$	N.D.	1.0	21
VII	pre IFN-γ	GM3C-2	1.9×10^6	N.D.	N.D.	—	$.19\times10^6$	N.D.	0.93	10

a) See Methods for extract preparation. Note that two methods were used for extracts. When cells were spheroplasted the "inside cell" amount was really inside material; however, when N.D. (not determined) is specified the "inside cell" amount and the "released after cell wall removal" were both part of "inside cell" amount—this type of extract involves glass beading cells without cell wall removal. Note that glass bead extracts without spheroplasting were always done for IFN-γ and preIFN-γ and that PBS buffer was used instead of 7M GHCl.

b) The specific activities of LeIFA and LeIFD were both assumed to be 10^8 U/mg protein for the calculations. A yeast culture contains about 100 mg of protein in the culture at an Abs660 = 1.

c) See Methods for spheroplasting procedure.

d) Abs of culture at which assay done.

e) The percent secretion is the percent "released after cell wall removal" plus the percent "outside cell". When spheroplasting was not done the "percent of activity secreted" does not include this cell wall secretion activity and the percent is lower (maybe 1/2) than it actually should be.

The first compartment is inside the cell. This fraction is measured by making a cell extract after the cell wall is removed and is defined as interferon activity that is not secreted. The other two compartments are the medium (material completely separate from yeast cell) and the activity released from the cells after cell wall removal using zymolyase. Both the medium fraction and fraction released after cell wall removal represent the total secreted material. Alternatively when cell walls were not removed (see Methods), "inside cell" activity also includes the secreted activity present in the cell wall.

It should be noted that (pre D/A) LeIF A is a mature LeIF A gene with a hybrid signal peptide sequence (see Figs. 3 and 4). This construction was made using the DdeI restriction site common to both preLeIF D and preLeIF A. Fig. 4 shows this DdeI site at amino acid -10. The underlined amino acids represent differences between the amino acid sequence of preLeIF D and preLeIF A. The hybrid (pre D/A) LeIF A is more like preD than preA. Both mature LeIF A and LeIF D genes (constructions I and III) were expressed in the yeast as 1.0 percent of the total cellular protein. The wrong orientations of all genes inserted into YEplPT did not express interferon activity. For these two mature genes as well as the mature IFN-γ gene, no secretion occurred. However, when presequences were used on these genes, all three protein products were found in the media as secreted products.

As shown in Table 1, levels of interferon secretion vary from one gene to another with immune interferon giving the highest level of secretion. Secretion into the medium of this interferon varies from 10–21 percent; however, another 10–21 percent may be in the cell wall (not determined). By comparison of the production of interferon for constructions VI and VII, it is interesting that yeast tolerates 50 bp of preIFN-γ 5'-flanking cDNA sequence; even though such an intrusion between the yeast promoter and structural gene results in about a 5-fold decrease in expression. Pre LeIF D and (pre D/A) LeIF A secrete from 3–5 percent into the cell wall and medium combined at an A_{660} = 1; however, when (pre D/A) LeIF A/20B–12 was grown to A_{660} of 3–4, 8 percent secretion into the medium was observed. Although the percent in the cell wall was not determined at this higher A_{660}, as much as 16 percent of the activity was probably secreted.

Growth Curve and Interferon Production in the Medium from a Yeast Containing the (pre D/A) LeIF A Gene

Two interferon-producing yeast were investigated by further characterization. These were YEplPT–preLeIF A53t/20B–12 and YEplPT–LeIF A1/20B–12. The former contains two copies of construction IV (Fig. 3) in the EcoRI site of YEplPT and results in activity being inside the cell, in the cell wall, and outside the cell (medium). This two copy gene (in tandem – both in orientation for proper expression) containing plasmid was used instead of the single copy construction since it sometimes gave higher levels of interferon activity in the medium. However, more careful comparisons have shown this not to be the case (both express about the same). Furthermore, the transcript stops at the end of the first structural gene in the converter as previously discussed. Yeast strain 20B–12 was used since it has greatly reduced protease levels (24), which might be an advantage for obtaining undegraded protein (interferon) from the

DdeI site in DNA

Cleavage site

	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2
preA	Met	Ala	Leu	Thr	Phe	Ala	Leu	Leu	Val	Ala	Leu	Val	Leu	Ser	Cys	Lys	Ser	Ser	Cys	Ser	Val	Gly	Cys	Asp	
preD	Met	Ala	Ser	Pro	Phe	Ala	Leu	Leu	Met	Val	Leu	Val	Leu	Ser	Cys	Lys	Ser	Ser	Cys	Ser	Leu	Gly	Cys	Asp	
preD/A	Met	Ala	Ser	Pro	Phe	Ala	Leu	Leu	Met	Val	Leu	Val	Leu	Ser	Cys	Lys	Ser	Ser	Cys	Ser	Val	Gly	Cys	Asp	
preγ			Met	Lys	Tyr	Thr	Ser	Tyr	Ile	Leu	Ala	Phe	Gln	Leu	Cys	Ile	Val	Leu	Gly	Ser	Leu	Gly	Cys	Tyr	
preβ			Met	Thr	Asn	Lys	Cys	Leu	Gln	Ile	Ala	Leu	Leu	Leu	Cys	Phe	Ser	Thr	Thr	Ala	Leu	Ser	Met	Ser	

Fig. 4. Amino acid sequences of secretion signals. A comparison of the amino acid sequence of the signal prepeptide regions of human IFN-α1 (pre D), IFN-α2 (pre A), IFN-α1,2 (pre D/A), IFN-γ (pre γ), and IFN-β (pre β) is shown. The amino acids underlined represent differences between the amino acid sequences of pre A and pre D. The DdeI site indicates the junction of the D and A pre-sequences in preparation of the hybrid pre D/A pre-sequence. The cleavage site that is used in human cells is shown.

medium. The latter yeast contain construction III (Fig. 3) in YEplPT and express mature LeIF A inside the cell but do not secrete.

Fig. 5 illustrates an essentially identical growth curve for these two yeast strains in YNB+CAA (Trp$^+$ selective growth). Bioassays, done on the medium at various times during cell growth, demonstrate clearly that the pre-sequence on LeIF A is causing a release of interferon activity into the media. Without this pre-sequence essentially no activity is released. This has also been confirmed by "Western" gel analysis (11) of proteins from concentrated media. Labelled antibody reacts only with a LeIF A size protein in medium from a pre-LeIF A expressor and not from a mature LeIF A expressor (data not shown). It is also evident that levels of activity in the medium reach a maximum near stationary phase.

One possible explanation for this apparent secretion process is that the pre-sequence of interferon somehow makes the cells more susceptible to lysis during cell growth. This possibility was carefully examined by measuring levels of protein in the media at stationary phase. Both yeast media contained essentially identical concentrations of protein which showed identical patterns on SDS-gel electrophoresis. Thus a true secretion process must be occurring.

Purification of (pre D/A) LeIFA from the Medium and Cells

In order to determine the nature of the secretion process for (pre D/A) LeIF A into yeast medium, it was necessary to purify the protein product from the medium and cells separately. Since yeast are able to secrete this protein, processing of the amino-terminal end may occur in some manner during the process as occurs in mammalian cells. The object of further experiments was to determine the nature of this processing.

Cell extracts and media were obtained from 5 l fermentations and the cellular interferon was purified as described in Methods. It should be noted that cell extracts also contain cell wall secreted material since the cell wall was not removed prior to extract preparation. The medium interferon was first concentrated by diafiltration, purified by CM-52 ion exchange chromatography, followed by the last two steps for cellular interferon purification.

NH$_2$-terminal Sequence of (pre D/A) LeIF A Purified from Yeast Medium and Cells

Fig. 6 shows the results of sequencing the purified interferon. The sequence shown is that expected for (pre D/A) LeIF A if no processing occurs. The normal cleavage point of this interferon that is recognized by mammalian cells is shown. Two sequence runs were performed on two different purified samples from cells and media.

The protein sequence was interpreted by noting which PTH amino acid increased in each corresponding Edman cycle and then decreased in the following cycle. PTH amino acids that normally give low recoveries (cys, ser, thr, arg, his) were assumed when no increase in any other PTH amino acid was seen. The percentage of each form was estimated by comparing the areas of the interpreted residue with area from a standard mixture of PTH
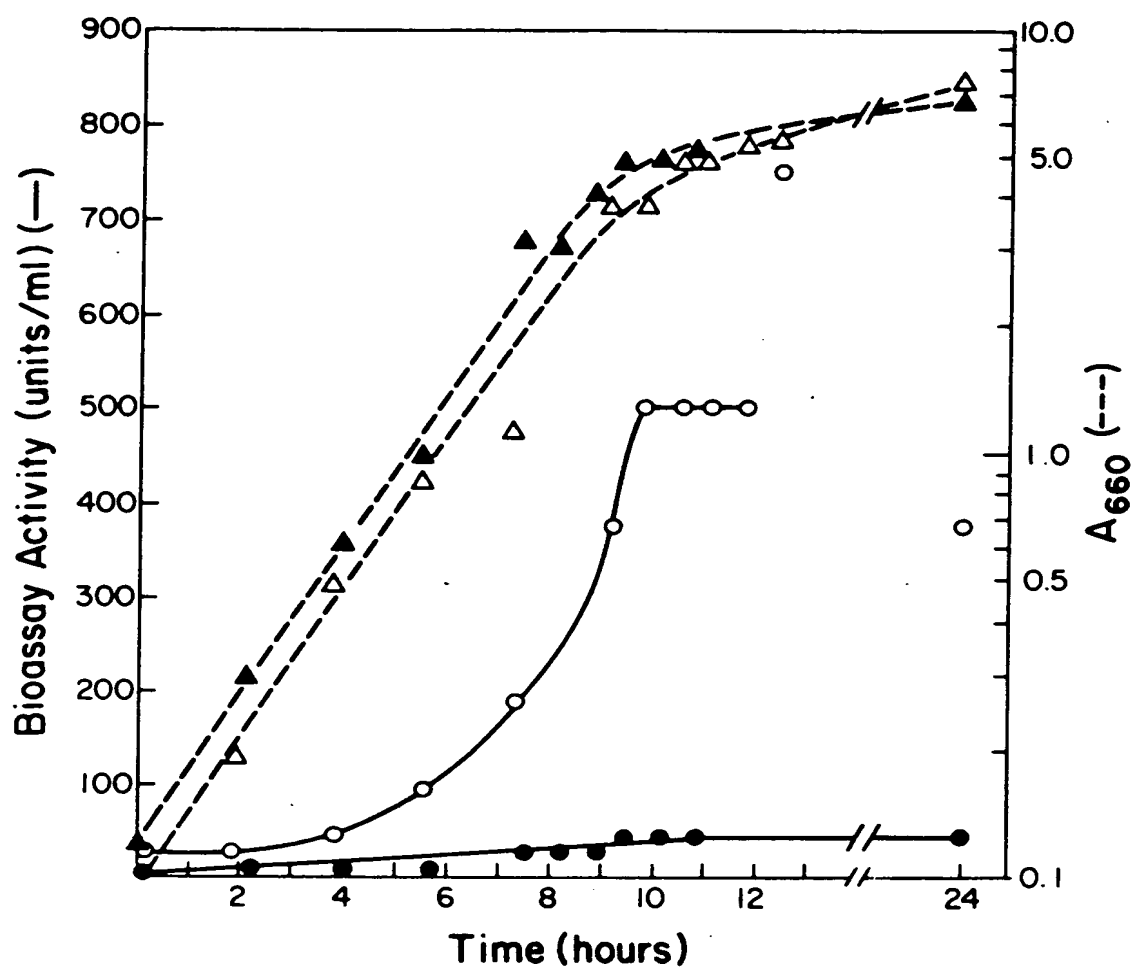
Fig. 3. Growth curve showing secretion into the medium. YEpIPT-LeIF A1/208-12 (▲ , ●) expresses mature interferon which remains within the cell; while YEpIPT-LeIF A33t/208-12 (△ , ○) expresses the pre-sequence containing gene and produces interferon activity in the medium.

|——————pre D/A secretion signal sequence——————————→|←——— mature LeIFA sequence

-23 -22 -21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1   1 2 3 4 5 6 7 8 9 10

met ala ser pro phe ala leu leu met val leu val leu ser cys ser ser lys ser ser cys val gly cys asp leu pro gln thr his ser leu gly

Growth Medium Interferon (10%):   None   36%   64%

Intracellular and Cell Wall Interferon (90%):   11%   55%   33%
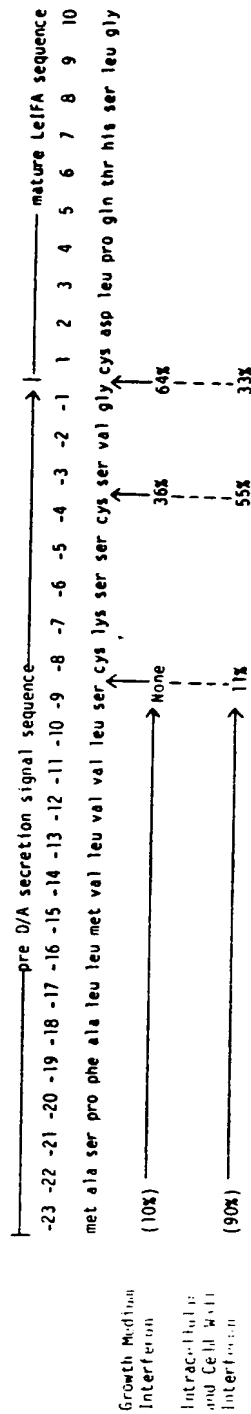
Fig. 6. Processing of (pre D/A) LeIF A by yeast. The 23 amino acid pre-sequence of (pre D/A) LeIF A interferon is shown as well as 10 amino acids of mature interferon sequence. Ten percent of the interferon made was found in the medium and 90 percent was cell associated (about 80 percent in the cell and 10 percent associated with the cell wall). So a small percentage (10 percent) of the 90 percent was also secreted interferon. The interferon from these two growth fractions (medium and cells) was processed as shown.

amino acids run on the same HPLC. Most of the forms were sequenced for 21 cycles or much further into mature LeIF A sequence than shown. An internal standard of Nor-Leucine was introduced in each chromatogram to assure that retention times were reproducible.

Figure 6 shows that most of the interferon in the medium was properly processed (64 percent) as done by human cells (13); however, another form (36 percent) containing three additional amino acids of pre-sequence was also present. The cellular interferon also contained these two forms, but in slightly different proportions, as well as a third form containing 8 amino acids of pre-sequence that was not detected in the medium. In no sequencing experiment was a full length pre-sequence ever seen suggesting that yeast process all of the pre-interferon in some manner. It is possible that sorting is a problem and that transport into other organelles occurs. Examination of the cellular location of these forms in the yeast cell versus the expressed mature form should give some insight into this possibility.

DISCUSSION

Using the isolated PGK gene (21), we have again demonstrated that the 5'-flanking DNA sequence of a highly expressed gene from yeast can be isolated and used as a portable restriction fragment for the expression of heterologous gene products in yeast. Careful comparisons of this promoter fragment and an ADH1 promoter fragment (fragment no. 906, 22) have shown essentially identical expression levels of leukocyte interferon D (data not shown).

In previous experiments, we suggested the need for a yeast 3'-flanking sequence for termination of transcription (22). By the size of the transcripts, it appeared that the mRNA of the hybrid expression unit, 5'-ADH1 promoter-LeIF D-TRP1-3', was terminating in the yeast TRP1 gene terminator. Indeed further experiments demonstrate the need for this signal with little or no expression occurring if such a signal is not present adjacent to the gene (data not shown). Use of such a hybrid expression system should allow further insights into terminator function, factors involved in mRNA synthesis and stability, mRNA translatability, and possible implications of codon usage (4) on expression of heterologous genes.

Using a plasmid containing the PGK-derived hybrid expression system, we have demonstrated the expression of both mature and pre-forms of human interferon cDNAs. The mature forms lack the secretion signal sequences and produce interferon within the yeast cell. The pre-forms contain the secretion signal coding sequences and produce interferon activities both inside and outside the cell. The secreted interferons were found both in the cell wall and unattached to the cell in the medium. The interferon, from yeast expressing the (pre D/A) LeIF A cDNA, was purified from cells (containing both intracellular and cell wall secreted material) and from medium. Two forms of interferon were found in the medium (10 percent of the external interferon, one properly processed (+1, Fig. 6) and the other with three additional amino acids of pre-sequence (-3, Fig. 6). The second form may be demonstrating a difference in yeast versus human cell processing recognition or it may be the result of the hybrid signal sequence. Further purification and sequencing of pre LeIF D should address this possibility.

It might also be possible that human cells make some of this -3 form that has not been noticed in interferon preparations.

Three forms of interferon were isolated from cells representing 90 percent of the expressed interferon. One form (33 percent) was properly processed (+1, Fig. 6), a second form (55 percent) contained 3 additional amino acids (-3, Fig. 6), and a third form (11 percent) contained 8 additional amino acids (-8). The last form was not seen in medium and a full length pre-sequence was never observed. However, it is possible that this peptide lacks interferon activity and thus was lost during purification. Therefore yeast appear to process both the secreted and nonsecreted interferon. The reason for only 10–20 percent secretion (20 percent if cell wall bound material included) instead of higher secretion levels may be due to a sorting problem associated with the heterologous signal. Interferon may be going into various organelles instead of through the plasma membrane. To examine this, we plan to compare cellular locations of interferon from both mature and pre-interferon producing yeast.

It would also be interesting to see if the interferons are secreted by yeast using the exocrine-like pathway which has been defined in yeast by sec mutants (28). Unlike other enzymes secreted by this pathway, the mature leukocyte interferons lack N-glycosylation and are probably not glycosylated when secreted by yeast. The effect of tunicamycin, which inhibits acid phosphatase and invertase secretion (25), would also be of interest. Perhaps a new secretion pathway might be defined by leukocyte interferon secretion from yeast.

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14. DOUBLE-STRAND BREAKS AND GENETIC RECOMBINATION

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Recombination of a plasmid with the yeast genome after transformation is stimulated by introducing a double-strand break in the plasmid molecule within a DNA fragment that is homologous to the chromosome. Transformation with a plasmid containing two double-strand breaks (a gap) within a single yeast DNA fragment results in a recombination event in which the gap is repaired using chromosomal information as the template. We have shown that gapped plasmids that contain an autonomous replicating sequence are also repaired and either a replicating or integrated plasmid is produced. We propose a model based on these observations that explains the repair of double-strand breaks and that postulates that double-strand breaks are important initiators of genetic recombination in yeast.

INTRODUCTION

The process of homologous genetic recombination requires 1) synapsis, 2) strand exchange and 3) resolution. We are using DNA transformation of yeast cells as a model system to study the various processes involved in recombination (9). The ability to experimentally manipulate plasmid DNA facilitates these studies. We have examined the interactions of both replicating and non-replicating plasmids with homologous chromosomal sequences. Non-replicating circular plasmids require integration into the chromosome to be stably inherited and the plasmids integrate by homologous recombination into the genome (3,10). In about one-third of the transformants, yeast sequences on the plasmid are substituted for the chromosomal sequences without integration of the entire plasmid (3). This class is functionally a gene conversion event. Replicating plasmids are maintained exogenously and consequently show an unstable phenotype for selectable markers on the plasmid.

We have previously shown that circular plasmids linearized by a restriction enzyme cleavage within a yeast sequence recombine into the homologous region of the genome (9). The double-strand break produced by the restriction enzyme cleavage stimulates recombination between 10- and 1000-fold over that found for circular integrating plasmids. We have also shown that a gap (two double-strand breaks) within a yeast segment is repaired using chromosomal sequences, and the resultant integrated plasmid is flanked by two complete direct repeats of the target sequence. These observations have led to methodologies for the directed integration of yeast plasmids into desired

regions of the genome, for the recovery of chromosomal alleles, and for mapping the position of alleles within cloned segments (10). We propose that double-strand break repair provides a general model for recombination in fungi.

RESULTS

Circular molecules integrate by homology. Integrations into the yeast genome occur by homologous recombination. The plasmid pWJ12 contains a. 1.8kb EcoRI - BamHI fragment from the sup3+ region of chromosome XV as well as a 1.7kb BamHI fragment of the HIS3+ region from the other side of the centromere on chromosome XV (Figure 1). After transformation of 10^8 cells with 10ug of DNA we observed 16 HIS3+ transformants. Colonies with integrated plasmid were detected by standard yeast filter colony hybridization using bacterial plasmid sequences as a probe (3). HIS3+ colonies that failed to hybridize with the probe presumably became HIS3+ by a gene conversion event. These colonies (4 of the 16) were not further analyzed. Southern blot analysis (14) was performed after isolating DNA from twelve transformants to determine the site of plasmid integration. Integration into either the sup3 or the HIS3 region produced a characteristic restriction fragment after digestion of the genomic DNA with the restriction enzyme SalI (see Figure 1). The fragments were detected by hybridization of genomic blots with labeled plasmid as a probe. Of 12 transformants examined, 4 contained the plasmid integrated at the HIS3 region and 8 at the sup3 region (Figure 2). The larger number of integrations into the sup3 region reflects an as yet undetermined but reproducible property of the sup3 region.

Double-strand breaks stimulate recombination. The enzyme, XhoI, creates a double-strand break outside the coding sequence of the HIS3+ gene but within the HIS3+ fragment. After transformation with 10ug of XhoI-cut plasmid DNA 140 transformants/10^8 cells were detected. Approximately 90% contained pBR322 sequences and 12 were analyzed by Southern blots. The appearance of a genomic 24kb SalI fragment in each transformant after probing with radioactive pBR322 sequences indicated that the plasmid had integrated into the HIS3 region (data not shown). The creation of the double-strand break both stimulated recombination (10-fold more than circles in this example) as well as directed integrations to the HIS3 region.

Gapped molecules are repaired. The plasmid pWJ12 was digested with SmaI to create a 1200bp gap within the sup3+ region. Forty-three transformants were recovered after transforming with 10ug of DNA/10^8 cells. The 3-fold stimulation of transformation is low presumably due to the small amount of homology left near the border of the sup3+ DNA and pBR322 sequences (170bp). All transformants that contained plasmid sequences were integrated at the sup3 region as demonstrated by Southern blots. The gap was repaired and the sup3 regions flanking the plasmid sequence were both full length. We have previously shown that chromosomal information was used as template for the gap repair (10).

We constructed a plasmid that contains the HIS3 region as well as the 2kb SalI-XhoI HIS3+ gene and the autonomous replication sequence arsl. The plasmid was made his3⁻ by creating a deletion between the two BglII sites within the HIS3+ gene. Therefore, digestion with BglII generates a 60bp gap. When yeast is transformed with BglII-cut pSCS11 and selection is made for HIS3⁻, transformants arise only if the gap is repaired from the

(A)

(B)

(C)
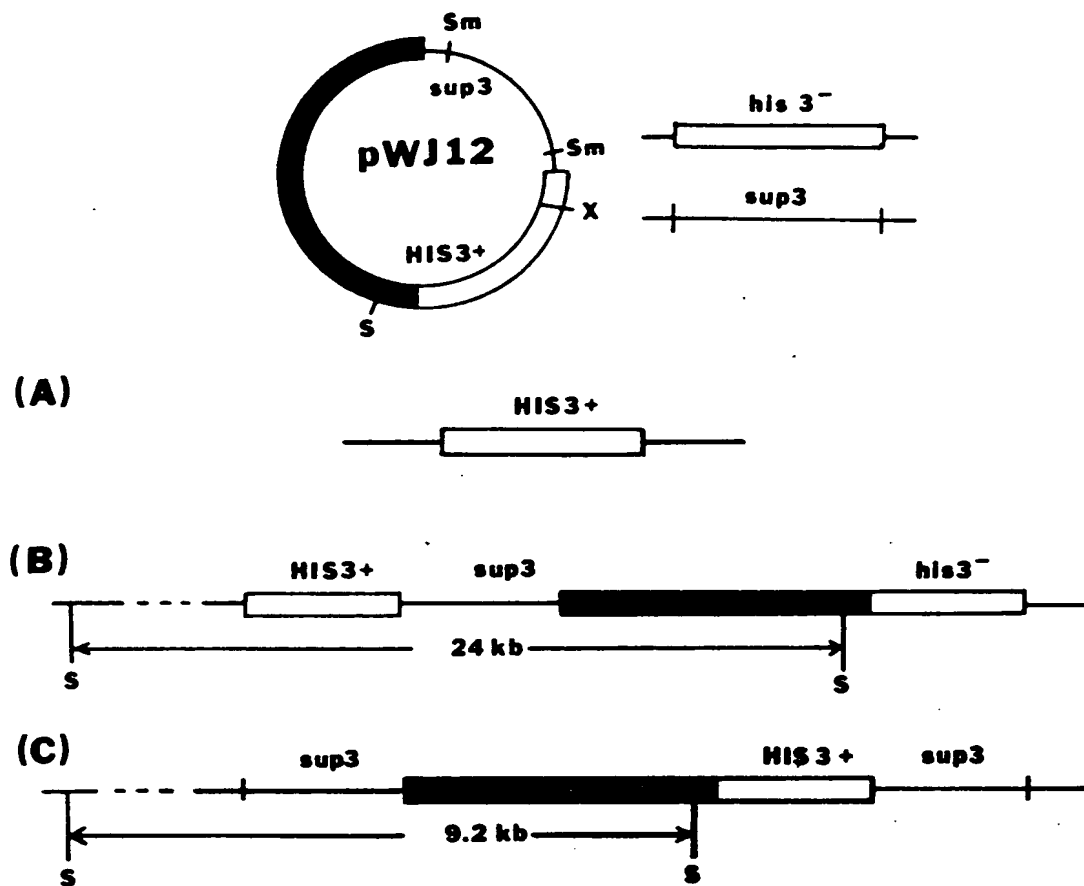
Figure 1.   Integration of pWJ12 into the yeast genome. The 7.4kb plasmid, pWJ12, contains DNA homologous to the two yeast regions <u>HIS3</u> and <u>sup3</u> (shown to the right) cloned in pBR322. <u>HIS3</u>+ transformants can arise in three ways: (A) gene conversion of HIS3+ information from pWJ12 into the genome without integration of the vector sequences, (B) integration of pWJ12 by a single crossover at <u>his3</u>⁻ or (C) integration of pWJ12 by a single crossover at <u>sup3</u>.   Restriction sites <u>Sal</u>I, <u>Sma</u>I and <u>Xho</u>I are designated as S, Sm and X respectively.
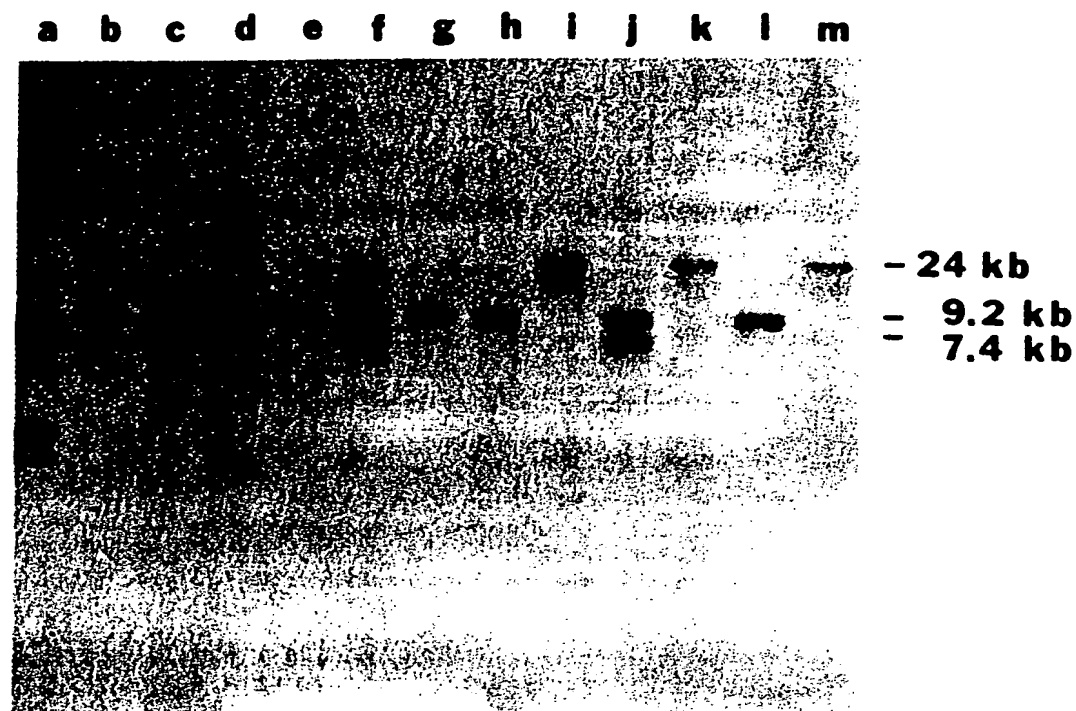
Figure 2.   Genomic blot of pWJ12 transformants.   DNA was isolated from 12 strains that had been transformed with circular plasmid DNA from pWJ12. Approximately 0.5ug of total genomic DNA was digested with the restriction enzyme SalI and electrophoresed for 16 hours in a 0.7% agarose gel at 75 volts in tris borate buffer as described previously (12).   The DNA was blotted and hybridized with ra.. . .ly la.... p..... (12).   Lane a shows the position of linear pWJ12 ... . s... . ... plasmid has integrated at sup3 in strains represented .. . .,f,g,h,j and l as indicated by the 9.2 kb band.   Integrations at his3 are indicated by the 24 kb band (lanes d,i,k and m).   The presence of the additional band at 7.4 kb in lanes d,f and j is indicative of multi... .. .. int.grations (9).

chromosomal HIS3 region. Re-ligation of the gapped plasmid would result in a his3⁻ transformant (Figure 3A). After transformation with BglII-cut linear molecules, both stable and unstable transformants are observed at approximately equal frequency. The stable transformants contain the repaired plasmid integrated at the HIS3 region (Figure 3B). The HIS3+ transformants exhibiting the unstable phenotype are due to molecules that interacted with the chromosome and repaired the gap without subsequent integration (Figure 3C). The generation of unstable gap-repaired molecules at such a high frequency indicates that they are one of the normal products of the molecular interaction between the incoming plasmid and the chromosome - the other potential product being an integrated plasmid.

DISCUSSION

It is clear from our studies that free DNA ends are recombinogenic. The properties of gapped plasmid integration demonstrate that an efficient repair mechanism exists for resolving these structures. These. results suggest new methods for the manipulation of plasmids during yeast transformation (10). We propose a mechanism for genetic recombination based on the idea that double-strand breaks and their subsequent repair can explain the observed fungal data for gene conversion and crossing over. As a detailed model will appear in the future, in this communication we briefly outline some aspects of this proposal.

The initial steps in the repair of gapped molecules are presented in Figure 4. In this model both ends invade the target double helix in a concerted fashion. We imagine that the initial invasive event actually occurs at either end of the double-strand gap. After the initial invasion the other free end has a high probability of invading its target site due to the close proximity of its region of homology. Each invading 3' end primes the repair synthesis necessary to fill in the gapped region using the existing chromosomal sequence as template. At the end of the replication-repair event, the repaired region is flanked by two Holliday structures (4). Theoretically, each Holliday structure could be independently resolved to yield two separate duplexes that have either crossed over with respect to each other or have resolved without a crossover. If each of the two Holliday structures in Figure 4 are resolved differently - crossover and non-crossover - the net result is a single crossover and the plasmid integrates. If both are resolved similarly, no plasmid integration occurs. However, the heteroduplex present at the initial site of pairing of the invading strand may result in gene conversion.

We have generalized this view of gapped plasmid integration in a model for genetic recombination based on double-strand break repair. In brief, the initiation of genetic recombination occurs at or near selected sites and is initiated by a double-strand break. The polarity of gene conversion suggests the existence of specific initiation sites. Exonucleolytic degradation exposes single strands which invade homologous chromosomal sequences; repair synthesis ensues and leads to the double Holliday structures shown for plasmid integration in Figure 4. Earlier investigators including Sobell and Holliday (5) have postulated such intermediates for recombination. It is noteworthy that Leslie Bell and Breck Byers (personal communication, have observed DNA structures during meiosis in yeast that are consistant with the view that Holliday structures are frequently found in pairs separated by 300-800 base pairs.
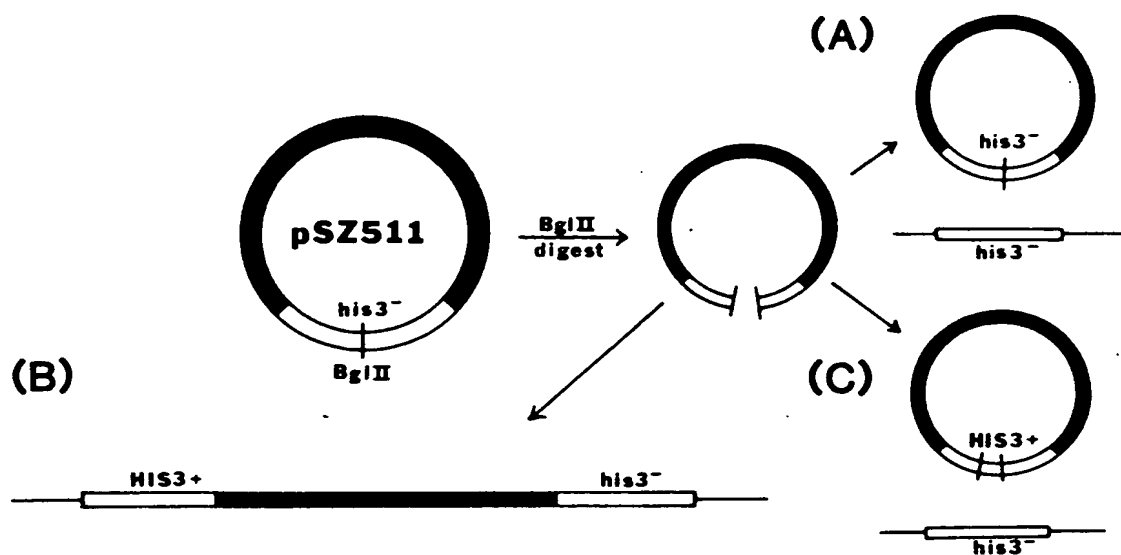
Figure 3.   Transformation with gapped pSZ511.   The LEU2+ arsl his3⁻ plasmid
pSZ511 contains an approximately 60 base pair deletion within the HIS3+ DNA
sequence and is, therefore, his3⁻.   The LEU2+ arsl pBR322 portion of the
plasmid is indicated by the thick solid black line.   The plasmid was
linearized with BglII and transformed into a his3⁻ yeast cell.   If
re-ligation of the plasmid occurs, the cell, remains his3⁻ due to the
deletion of the DNA between the two BglII sites (A).   Gap repair and
integration as described in the text could occur and a stably integrated HIS3+
plasmid is the result (B).   Lastly the plasmid could interact with the
chromosomal his3 region, repair the gap without subsequent integration, and
result in an unstable HIS3+ phenotype due to the repaired autonomously
replicating plasmid (C).

Holliday Structure   **Holliday Structure**
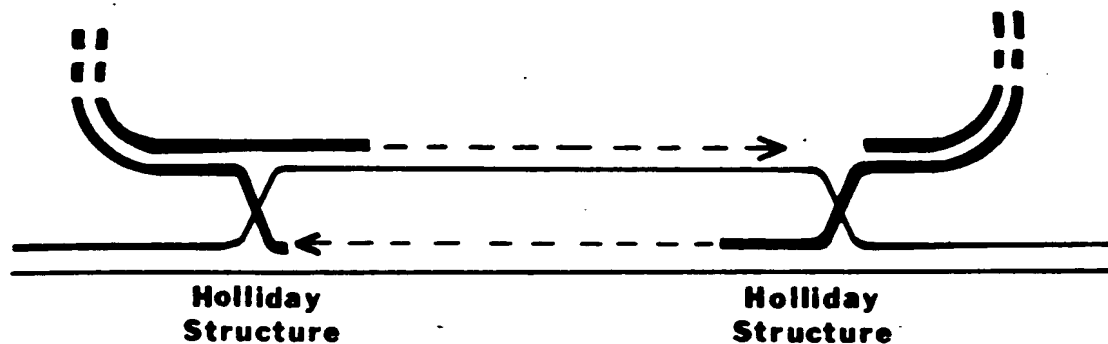
Figure 4. Model for the repair of a double-strand gap. The thin lines represent chromosomal duplex DNA and the thick lines represent plasmid DNA. The dashed lines represent repair synthesis. The incoming plasmid DNA is shown invading the homologous region of the chromosome. Th... ...d can ... as a primer for repair synthesis. After repair two Holliday structures are formed. Resolution of the ... liday structures results in ... integration of the plasmid or a donation of a small portion of plasmid sequences (depicted as a thick line) without the integration of the entire plasmid.

After the formation of the two Holliday structures, rotary diffusion (7) of the structure may occur. This feature accommodates the doubly heteroduplex regions necessary to generate aberrant 4:4s observed in <u>Ascobolus</u> and <u>Sordaria</u> (6,11). For yeast, in which there is a paucity of aberrant 4:4s (1), we postulate that such rotary diffusion cannot occur. The generation of 6:2 and 2:6 tetrads is postulated to occur by the repair of a gapped region. 5:3 and 3:5 segregations are accomodated by failure to repair the heteroduplex region created by strand invasion. There are two notable features of our model that fit with observations of conversion in yeast tetrad data. One is that the 6:2 and 2:6 tetrads, which rarely favor one parent versus the other (1), can arise wholly from degradation of either chromatid and the subsequent repair of the duplex. This event does not require non-random mismatch correction as implied in other models (8). Secondly, when Fogel and his co-workers examined post-meiotic segregations at the <u>arg</u>4 locus, they found that the associated crossover could occur on either side of the conversion event (1). The double-strand break repair model postulates the existence of two Holliday structures and thus accomodate this observation.

The features of the double-strand break repair model for recombination also make it an attractive model for HO-initiated mating type interconversion (2). The mating type system, however, puts an additional constraint on the crossover outcome - there can be no (or extremely rare) exchanges since this would cause lethal deletions of part of chromosome III.

ACKNOWLEDGEMENTS

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15. IDENTIFICATION OF CLONED GENES THAT COMPLEMENT THE rad50-1, rad51-1, rad54-3 and rad55-3 MUTATIONS IN YEAST

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Plasmids that complement the rad50-1, rad51-1, rad54-3 and rad55-3 mutations in yeast, have been isolated. They were obtained by transforming strains, carrying the leu2-112 leu2-3 alleles and the particular rad mutation, with YEp13 plasmids containing near random yeast DNA inserts. Rad$^+$ clones were identified among the Leu$^+$ transformants. Integration by targeting into the RAD55 locus showed that the rad55-3 complementing plasmid contained the actual RAD55 gene. BamHI fragments from each of the plasmids that complement rad50-1, rad51-1 and rad54-3, all of which lacked Rad$^+$ activity, were sub-cloned into the integrating plasmid YIp5 and the hybrid plasmids were used to transform a Rad$^+$ Ura$^-$ strain to Ura$^+$. By genetic mapping, the rad51 and rad54 subclones were shown to integrate at their respective loci. However, the rad50 subclones integrated at a site unlinked to the RAD50 locus. This suggests that no homology exists between this BamHI fragment and the RAD50 gene. Integration at the RAD54 locus of the rad54 subclone made the host cell Ura$^+$ but Rad$^-$; excision of the plasmid was shown to be X-ray inducible and to restore the Ura$^-$ Rad$^+$ phenotype. These results indicate that the BamHI fragment of the RAD54 plasmid is internal to the RAD54 gene. We can conclude also that the RAD54 gene is not essential as cells bearing a disrupted copy of this gene are able to survive.

Additionally, a plasmid carrying an amber suppressor has been isolated and characterized.

INTRODUCTION

The ability to repair DNA that has been damaged seems to be an important characteristic, common to all living organisms. Damage can arise spontaneously or can be induced by both physical and chemical agents; repair of the damage can be accomplished through different pathways, depending on, for example, the kind of damage or the physiological state of the cell.

In the yeast Saccharomyces cerevisiae evidence for the repair of potentially lethal damage has been provided by the isolation of a large number of mutant strains that are more sensitive than the wild type to the lethal effects of various mutagens, such as ultraviolet light, X-rays and methyl-methane-sulfonate (see (10), (14) and (15) for recent reviews). These mutants fall into more than 50 different complementation groups: one of them is known to define a photoreactivation gene (19); the rest are thought to be involved in dark repair. The fact that so many genes participate in dark repair implies that these processes are under extensive genetic control in yeast. Studies with double mutants revealed that these genes can be classified into three epistatic groups (4,9) which are believed to represent three different repair pathways: 1) Excision repair, carried out by the genes in the RAD1 group; 2) error prone repair, controlled by genes in the RAD6 group; and 3) recombinational repair, controlled by the genes in the RAD52 group. This last group of genes seems to be involved in several cellular processes as demonstrated by the fact that their mutants have complex phenotypes; not only are they defective in repair, but also they exhibit abnormalities in mitosis, meiosis, recombination and homothallic switching. However, very little is known about the function of these genes at the molecular level.

We have chosen to isolate these genes by cloning them into replicative plasmids, followed by in vivo and in vitro characterization. By such studies, we hope to determine if these genes are essential for the vegetative life of the yeast cell, how they are regulated and what are their products.

In this paper we describe the preliminary steps of these investigations, namely the isolation and characterization of plasmids that complement the mutations rad50-1, rad51-1, rad54-3 and rad55-3.

MATERIALS AND METHODS

Strains: The yeast strains used for transformation are listed in Table 1; they were constructed by standard techniques of yeast genetics (21). Unless otherwise stated, the rest of the strains were obtained from the Yeast Genetic Stock Center (University of California, Berkeley).

Table 1. Transformable strains used

Strain	Genotype	Source
XS131-5A	MATα rad50-1 leu2-112 leu2-3 ura3-52 trpl-289 his3-Δ1 lys1-1	D. Schild
XS133-3B	MATα rad51-1 leu2-112 leu2-3 ura3-52 trpl-289 his3-Δ1	D. Schild
XL4-22C	MATα rad54-3 leu2-112 leu2-3 ura3-52 trpl-289	this work
XL5-3C	MATα rad55-3 leu2-112 leu2-3 ura3-52 trpl-289 his3-Δ1 his5	this work

The Escherichia coli strains used were: HB101 (3), provided by A. J. Clark; JA300 (23), provided by J. Carbon, and DB6507 (a pyrF derivative of HB101), provided by D. Botstein.

Plasmids: YEp13 (5)is a derivative of the pBR322 plasmid of E. coli that also carries the LEU2 gene of S. cerevisiae and a fragment of the yeast 2 plasmid with its origin of replication. pBR322 confers resistance to ampicillin and tetracycline to the host bacterial cell.

YRp7 (23) is also a pBR322 derivative, carrying the yeast origin of replication ARS1 and the yeast TRP1 gene; it was provided by D. Botstein.

YIp5 (20) consists of pBR322 and the URA3 gene of yeast. This plasmid does not replicate autonomously in yeast but is able to transform it by integration; it was provided by D. Botstein.

Yeast pool 35 DNA was provided by K. Nasmyth; it was constructed in a similar way as that described by Nasmyth and Reed (17) but using the vector YEp13 instead of YRp7.

Media and Genetic Methods: Growth media and standard genetic procedures for yeast have been described elsewhere (21). Tests for auxotrophy were carried out in synthetic complete media lacking the particular requirement.

E. coli growth media have been described before (7). When necessary, ampicillin (60 µg/ml) and/or tetracycline (15 µg/ml) was added to the media.

Enzymes: Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and Bethesda Research Laboratories (Rockville, MD) and used as directed.

Plasmid extractions and purifications: Plasmid DNA from yeast was extracted by the method described by Nasmyth and Reed (17).

Two different methods were used to extract DNA from E. coli: for quick small-scale preps the method of Holmes and Quigley (12) was applied; when large quantities of plasmid were required, the procedure described by Birnboim and Doly (1) was used with minor modifications, scaled up to 1 liter cultures. Plasmid DNA was then purified by equilibrium density sedimentation in a CsCl-ethidium bromide gradient, as described by Davis et al. (7).

Transformation: Yeast transformation was carried out as described by Hinnen et al. (11) with minor modificatons.

E. coli strains were transformed using the procedure described in Davis et al. (7).

X-ray Treatment: The X-ray source and treatment used are described in Game and Mortimer (9), except that the dose rate used was 237.5 rad/sec and the standard dose was 57 Krad.

Electrophoresis: Electrophoresis was carried out in 1% agarose slab gels according to the method described by Davis et al. (7). Tris-borate buffer was used both in the gel and in the chambers.

RESULTS AND DISCUSSION

Isolation of plasmids

The pool 35 DNA was used to transform to Leu⁺ the yeast strains listed in Table 1. The resulting transformants were tested for resistance to X-rays. In the case of rad50, rad51 and rad54, about 0.1 to 0.2% of the Leu⁺ tested turned out to be Rad⁺; in the case of rad55, the proportion was in the order of 1% (Calderon, Contopoulou and Mortimer, manuscript in preparation). If one considers that the genome of S. cerevisiae has enough DNA (13,500 Kb, according to Lauer et al. (13)) to code for around 10⁴ genes, and that each of the plasmids of pool 35 carries on the average a 5-7 Kb insert (17), one would expect to find a particular gene represented at a frequency of about 0.05%. We find no simple explanation for the considerably high frequency obtained in the case of rad55.

In order to determine to what extent each plasmid restores the radiation resistance of the transformants to the wild type condition, haploid and homozygous diploid Rad⁻ strains carrying or lacking the plasmids were irradiated with increasing doses of X-rays. The resulting survival curves were compared with those of similar Rad⁺ strains. In all cases studied, the presence of plasmids improved the resistance of the strains to X-rays, although the degree of improvement depended on the particular plasmid studied (Calderon, Contopoulou and Mortimer, manuscript in preparation). In no case did the degree of resistance exceed that of the wild type; we interpret this to mean that once the wild type level of repair has been reached, an excess of RAD gene product has no influence.

A phenotypic characteristic of some of the diploid Rad⁻ strains is poor sporulation and/or low viability of spores (22). In contrast to the restoration of X-ray resistance, the presence of the plasmids does not seem to

improve these sporulation defects (Calderon, Contopoulou and Mortimer, manuscript in preparation). This result might be explained, at least partially, by the fact that the plasmids are lost very often during meiosis: only 20–60% of the resulting spores retain them.

Restriction Maps

Plasmid DNA was obtained from each yeast strain and used to transform E. coli strain HB101 to ampicillin resistance and leucine autotrophy. Plasmid DNA was extracted and purified from the transformed clones. The purified DNA was then digested with EcoRI and the resulting fragments were separated by agarose gel electrophoresis. Those plasmids having the smallest insert (one for each RAD gene) were chosen for subsequent studies. Detailed restriction maps of each are shown in Figure 1.

In order to demonstrate that the purified plasmids were able to retransform the yeast strains from which they were isolated, and to determine if there was any cross complementation, the plasmids illustrated in Figure 1 were used to transform each of the Rad⁻ transformable strains (Table 1) to Leu⁺. When tested for X-ray sensitivity, all were Rad⁺ when the plasmid used carried the same putative RAD gene as that mutated in the recipient strains. Conversely, all Leu⁺ transformants remained Rad⁻ when the other plasmids were used.

Integration of Plasmids into the Genome

Complementation of a mutation by a plasmid may result from plasmids carrying the gene in question or another gene capable of "suppressing" either genetically or metabolically, the mutant phenotype. In the course of this investigation we have, in fact, isolated such a suppressor-bearing plasmid (to be described later) which complements the rad50-1 mutation. It therefore became essential to prove the identity of the cloned RAD genes by means of homologous integration into the yeast genome. It is commonly accepted that integration at a certain locus demonstrates that the plasmid carries that particular gene.

In view of the restriction maps, two different approaches were chosen to pursue the integration:

Integration of the rad55-3 complementing plasmid

As shown in Figure 1, the plasmid [YEp13-RAD55-13C] has a unique BamHI site. According to the observation of Orr-Weaver et al. (18), cleavage of this plasmid at the BamHI site would increase the efficiency of its integration within a chromosomal DNA region homologous to the yeast DNA that was cut.

About 25 g of the plasmid [YEp13-RAD55-13C] were digested with BamHI and used to transform the strain XL5-3C (Table 1) to Leu⁺. Out of 35 transformants studied, 3 (INT55-11, INT55-12 and INT55-30) failed to show loss of the Leu⁺ Rad⁺ characters indicating that the plasmid had been integrated. Strain INT55-11 was then crossed to strain XL39-5A (MATa rad55-3 leu2 ura3(and/or 4) met8 trpl-289 arolD his3(and/or 5), constructed by us).

RESTRICTION   MAPS
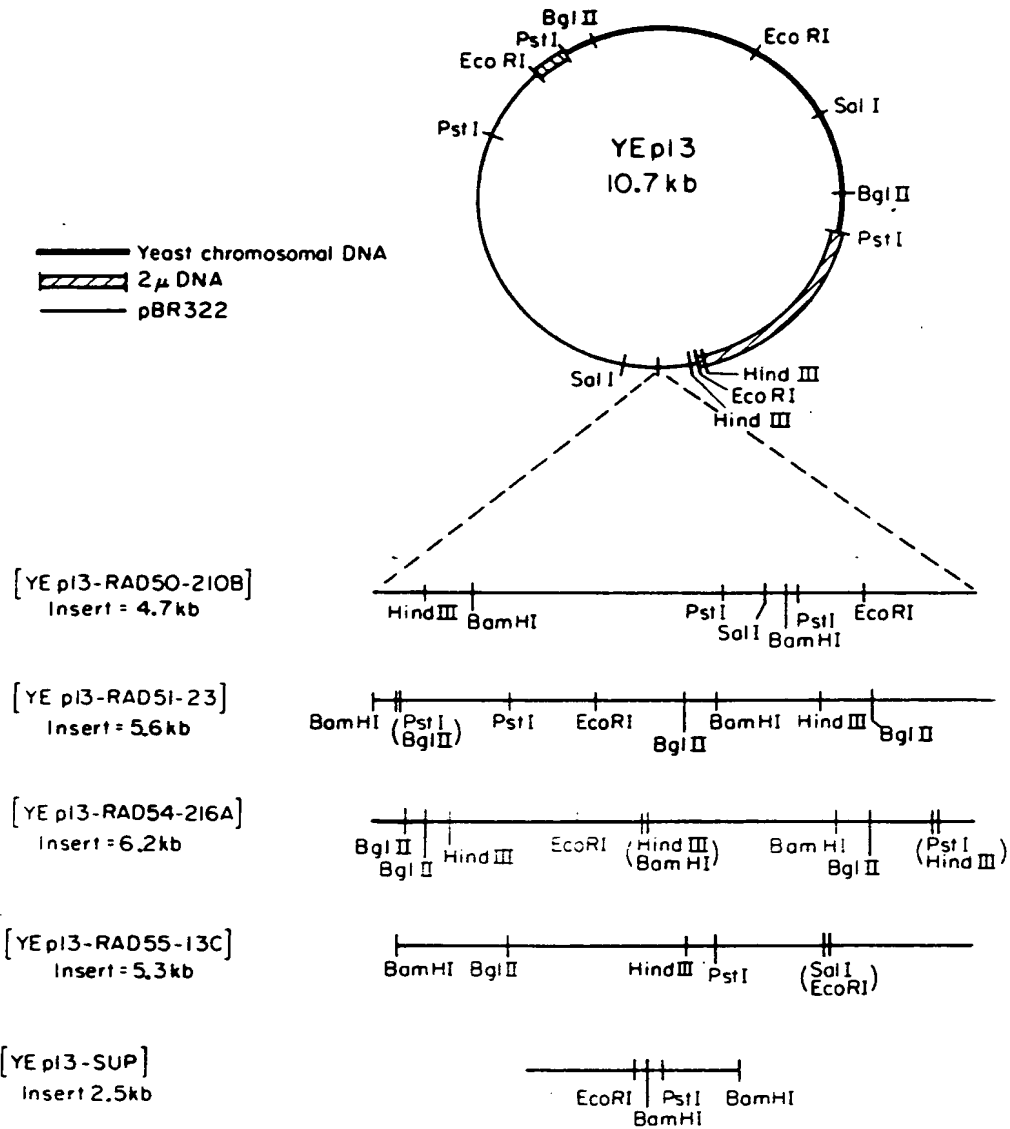
Figure 1. Restriction maps of the plasmids.

The resulting diploids are, therefore, homozygous for rad55-3 and leu2-112 leu2-3 but contain an integrated copy of the wild type allele of both of these genes. The genes RAD55 and ARO1 are located on the right arm of chromosome IV and are separated by a distance of 42.9 cM (16). About 60 asci derived from this cross were micromanipulated; only 52% of the spores were viable. Analysis of the asci containing 3 or 4 viable spores revealed that, as expected, both leu2 and rad55 markers segregated 2+ : 2- and all tetrads were parental ditypes; they mapped at a distance of about 40 cM from ARO1 (11 parental ditypes (PD): 1 non-parental ditype (NPD): 17 tetratypes (T)). These results indicate that the integration has taken place at the RAD55 locus and, consequently, that the RAD55 gene is present in plasmid [YEp13-RAD55-13C].

Integration of the rad50-1, rad51-1 and rad54-3 complementing plasmids

As shown in Figure 1, each of these plasmids has two BamHI sites that flank yeast DNA segments of 3 Kb, 3.4 Kb and 1.5 Kb, respectively. By subcloning these fragments into the single BamHI site of the integrative plasmid YIp5, then transforming the appropriate yeast strains with these plasmids and finally mapping the sites of integration in the yeast genome, one could demonstrate whether or not homology exists between the cloned fragments and the respective RAD genes. Before proceeding with the integration, we found it useful to determine if these BamHI fragments per se have Rad+ function. This was carried out by subcloning them into the replicative plasmid YRp7; the BamHI fragments were excised and religated into the single BamHI site of YRp7. The ligation mixtures were used to transform the E. coli strain JA300 to Trp+ AmpR. Plasmid DNA was extracted from those transformants that were also TetS, using the "quick prep" method. This DNA was digested with restriction enzymes and the resulting fragments separated by agarose gel electrophoresis. The plasmids having the expected banding pattern for YRp7 plus the respective BamHI fragment, in the same orientation as in the original plasmids, were called [YRp7-RAD..-BB]; one each from RAD50, 51 and 54 was chosen for subsequent studies. Plasmid DNA was extracted, purified and used to transform the appropriate Rad- strains to Trp+. In all three cases, the transformants tested were still Rad-, indicating that the Rad+ function is totally or partially outside the BamHI fragments.

The BamHI fragments were next subcloned into YIp5 in a way similar to that described for YRp7, except that the E. coli strain used for transformation was DB6507, and Ura+ AmpR transformants were selected. The plasmids obtained were called [YIp5-RAD..-BB] and were used to transform the yeast strain DBY947 (MAT ura3-52 ade2-101, provided by D. Botstein) to Ura+. Integrants were called INT50-.., INT51-.. and INT54-.., respectively.

So far, 32 different INT50 strains have been analyzed. All were Ura+ Rad+. Crosses by XS132-1A (MATa ura3-52 rad50-1 his3 leu2 trp1-289, provided by D. Schild) revealed that, in all cases, the ura and the rad markers segregate 2+ : 2- but in an independent fashion, meaning that the integration has not taken place at the RAD50 locus. This result suggests that the cloned fragment lacks homology with the RAD50 gene.

Two different hypotheses could explain these findings: 1) The [YEp13-RAD50-210B] plasmid carries a gene that is able to complement rad50 mutations only when present in a high copy number, and 2) the insert of the rad50-1 complementing plasmid is made up of at least two Sau3A fragments which ligated together in the process of constructing the yeast pool but are normally separated in the genome; the BamHI fragment would carry a part that is neither homologous, nor continuous to the RAD50 gene. Presently we are working on these hypothesis.

When the plasmid used for transformation was [YIp5-RAD51-BB], seven integrants were obtained and all were Ura$^+$ Rad$^+$. One, INT51-B, was crossed to strain XS134-4B (MATa ura3-52 rad51-1 leu2 his3 trp1, provided by D. Schild). The analysis of 18 tetrads derived from this cross revealed that URA3 and RAD51 were closely linked: all of the tetrads were parental ditypes. The integrant INT51-B was also crossed to strain 3971-5B (MATa gal10 SUC mal trp1 ura3 ura4 met8 ade5,7 leu2 lys1 arolD ilv1 can1). The genes RAD51 and ILV1 are located on chromosome V, about 11 cM apart (16). The results of the analysis of 15 tetrads showed that now URA3 and ILV1 were also linked (9 PD: O NPD : 6 T). We conclude that the integration has taken place at the RAD51 locus and that the plasmid that complements rad51-1 carries the RAD51 gene.

In the case of the INT54 transformants, two of them (INT54-A and INT54-D) were found to be Ura$^+$ Rad$^+$, while the other two (INT54-B and INT54-C) were Ura$^+$ Rad$^-$. Taking into account that the 1.5 Kb fragment carried by [YIp5-RAD54-BB] has no Rad$^+$ activity (as previously described), a possible explanation for the occurrence of Rad$^-$ transformants would be that the BamHI fragment is totally internal to the RAD54 gene; its integration at the RAD54 locus would then give rise to two incomplete RAD54 genes, separated by the YIp5 sequences. This hypothesis implies that in INT54-B and INT54-C the integration occurred at the RAD54 locus. This in fact was shown to be the case: when INT54-C was crossed to strain X4004-3A (MATa ura3 lys5 trp1 met2), a distance of 4.5 cM between the LYS5 and the URA3 loci was estimated (30 PD : O NPD : 3 T). According to J. Game (unpublished results), LYS5 and RAD54 are closely linked. In addition, this cross yielded only parental ditypes for the rad and ura markers.

When INT54-A, one of the Ura$^+$ Rad$^+$ transformants, was crossed to X4004-3A, the lys5 and ura3 markers segregated independently (3 PD : 1 NPD : 11 T), indicating that the integration has not occurred at the RAD54 locus. This might be explained by a gene conversion event between the URA3 allele on the plasmid and the chromosomal ura3 allele, or by integration of the plasmid at ura3. The latter explanation is unlikely because the mutation ura3-52 is thought to be a deletion and rarely undergoes homologous recombination (20).

The fact that the RAD54 gene can be completely disrupted by the plasmid and the cell still survives strongly suggests that it is a non-essential gene. In order to investigate how such a disruption affects the response of the cells to X-rays, INT54-B cells were irradiated with different doses of X-rays and the survivals determined. The experiment was carried out at 23°C and 37°C as the rad54-1 mutation used throughout these studies is thermosensitive (9).

As can be seen from Figure 2 (top), the same survival curve was obtained
at either temperature. Furthermore, the survival curves closely paralleled
that obtained with cells carrying the rad54-3 mutation, when incubated at 37°C
after irradiation, except for the resistant "tail" exhibited at high doses.
Such "tail" effects are characteristic of mixed populations consisting by, in
large, of sensitive cells with a small proportion of resistant ones. The most
likely origin of the resistant subpopulation is by excision of the integrated
plasmid, yielding back the original Rad⁺ Ura⁻ cells. This was tested by
checking the Ura phenotype of the survivors. As can be seen in Figure 2
(bottom), the proportion of Ura⁻ colonies increased with the dose of X-ray,
until they made up almost 90% of the surviving population. Further confirma-
tion for the occurrence of an excision event was obtained by showing that,
indeed, all the Ura⁻ clones, when tested for X-ray sensitivity, were again
Rad⁺.

Moreover, this excision seems to be induced by X-rays, as shown by the rise
of sectored colonies Ura⁺/Ura⁻ among the surviving ones (Figure 2, bottom).
Sectored colonies probably result from excision events occurring after
surviving cells have divided. If no induction takes place, one would expect
the proportion of sectored colonies to diminish with increasing doses, since
the subpopulation of cells from which they derive, are being killed
preferentially. The fact that the proportion increases rather than diminishes,
would indicate that the excision is being induced. At high doses almost all
the sensitive cells would have been killed; this would explain the eventual
decline of the proportion of sectored colonies.

Isolation of a Suppressor

While testing the Leu⁺ transformants, obtained with the pool 35, for
other markers present in the transformable strains (Table 1), we found that a
relatively high proportion of them (112/10,700 clones tested) were also Trp⁺.
Furthermore, in the case of the rad50 strain, most of the Rad⁺ (42/44) turned
out to be also Trp⁺. The genes RAD50 and TRP1 are located on chromosome IV,
but they are approximately 160 cM apart (16). Thus, the probability of having
both genes together on the same plasmid, particularly at the high frequencies
at which they were obtained, was thought extremely unlikely. A more likely
possibility was that we had cloned a nonsense suppressor. Since the
transformants were still Lys⁻ and the lys marker present in the transformable
strain, lys1-1, was an ochre mutation, we surmised that we were not dealing
with an ochre suppressor. In fact, both rad50-1 and trp1-289 were demonstrated
by standard procedures to be amber mutations and the plasmid that complements
them, [YEp13-SUP], was shown to suppress other amber mutations, i.e., aro1D-1,
met8-1, ade5,7 and trp-1 (Calderon, Contopoulou and Mortimer, manuscript in
preparation). A restriction map of this plasmid is shown in Figure 1.

The most likely origin of this suppressor is the yeast strain AB320
(MATa/MATα [HO trp5-2 ura3-1 (ural-1 ?) met4-1 ade2-1 leu2-1 lys2-1
canl-100]), which was the source of the insert DNA in pool 35. However, when
a haploid ho derivative of AB320 (AB320 was provided by B. Hall) was crossed to
strain 3971-5B that carries an assortment of nonsense alleles, no amber
suppressor could be detected among the segregants of this cross. The
possibility that AB320 carries a weak suppressor that is only active in high
copy number, as is the case with plasmid YEp13, cannot be ruled out.
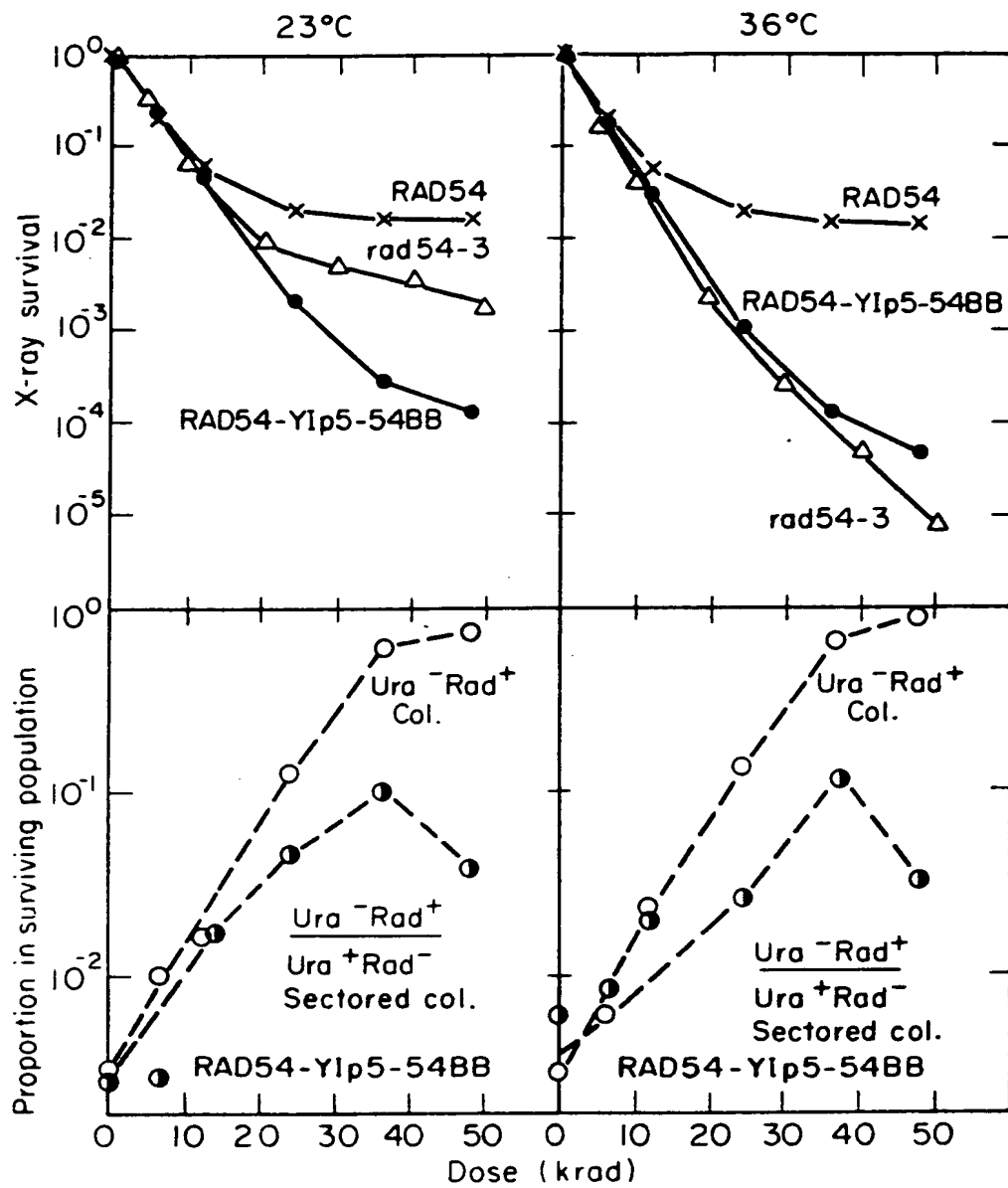
Figure 2. (top) Survival curves of strains carrying different alleles of RAD54; (bottom) proportion of Ura⁻ Rad⁺ and Ura⁻ Rad⁺/Ura⁺ Rad⁻ sectored colonies arising among the INT54-B (RAD54-YIp5-54BB) cells that survived different doses of X-ray.

Another possible explanation would be that the plasmid carries the PSI factor which is known to enhance the expression of low level suppressors (6). One would then expect a low level supressor to be present in all transformable strains. This, however, seems not to be the case as demonstrated by crossing the strain XS131-5A (Table 1) to the strain MT193/3b (MATa SUQ5 ade2-1 his5-2 lys1-1 trp5-48 can1-100 ura3-1 leu1 [PSI], provided by B. Cox). Analysis of tetrads derived from this cross showed that the presence of PSI does not affect the normal segregation of the rad and the trp markers.

Two other possible hypotheses about the origin of the suppressor remain unproved:

1. Plasmid [YEp13-SUP] could carry a mitochondrial tRNA gene that behaves as a suppressor in cytoplasmically-directed protein synthesis; the high copy number of mitochondrial genomes would explain the high frequency at which this plasmid is found in the pool. This seems an unlikely explanation, however. The wobble rules for codon:anticodon recognition in mitochondrial translation would preclude a mitochondrial tRNA from functioning as an amber-specific suppressor (2).

2. The fragment that contains LEU2 in YEp13 is known to contain also a gene coding for a tRNA (8). This might well be SUP53, a gene closely linked to LEU2 (16), some of whose mutations are known to suppress amber mutations.

Presently, experiments to elucidate which of these hypotheses may be the correct one, are being carried out.

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16. CLONING OF THE RAD52 GENE OF Saccharomyces cerevisiae

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The RAD52 gene of Saccharomyces cerevisiae has previously been shown to be involved in both recombination and DNA repair. Here we report on the cloning of this gene. A plasmid containing a 5.9kb yeast insert in the YEp13 vector has been isolated and shown to complement the X-ray sensitive phenotype of the rad52-1 mutation. From this insert various fragments have been subcloned into the YRp7 vector. A spontaneous integration event of one of the subclones has been genetically mapped to the chromosomal location of RAD52, indicating that the structural gene has been cloned. A 1.97kb BamHl fragment subcloned into YRp7 in one orientation complements the rad52-1 mutation, while the same fragment in the opposite orientation fails to complement. Various other subclones indicate that a BglII site, within the BamHl fragment, is in the RAD52 gene. This BglII site has been deleted by S1-nuclease digestion and the resulting deletion inactivates the RAD52 gene. BAL31 deletions from one end of a 1.9kb Sall-BamHl fragment have been isolated; up to 0.9kb can be deleted without loss of RAD52 activity, demonstrating that the RAD52 gene is approximately 1kb or less in length.

INTRODUCTION

Several different DNA repair pathways have been identified in yeast, including a presumptive recombinational repair pathway (reviewed in ref. 7 and 12). The RAD52 gene is one of at least eight genes (the RAD52 epistasis group) already identified which are thought to be involved in recombinational repair (5,7). The rad52-1 mutation was originally identified as causing extreme sensitivity to X-rays, but only slight sensitivity to U.V. light (19). Strains carrying this mutation have also been shown to be partially or completely defective in meiosis and sporulation (5,6,18,27), meiotic recombination (6,18,27), mitotic gene conversion (6,18,25,27), double strand break repair (10,21), homothallic switching (13), and maintenance of chromosome stability, resulting in chromosome loss (15). In addition, vegetative cells with the rad52-1 mutation have been shown to have elevated rates of spontaneous mutation and rad52/rad52 cells in meiosis have been shown to accumulate single

strand breaks during premeiotic DNA synthesis (22). If one assumes that double strand breaks and other X-ray induced DNA lesions are repaired by a recombinational mechanism (20), it is possible to account for most of the phenotypes associated with the rad52-1 mutation by its defect in recombination.

Although many phenotypes of the rad52-1 mutation have been observed, no known enzymatic or structural activity of the RAD52 gene product is known. In order to study the RAD52 gene at the molecular level, we have cloned this gene. The cloning of E. coli DNA repair genes, such as RECA and LEXA, has already been very important in isolating and characterizing the primary products of these genes and in studying their transcriptional regulation (reviewed in ref. 23). The cloned RAD52 will be used to determine whether this gene is transcriptionally regulated and if so, what are the inducers of transcription. The cloned gene is currently being used to identify and isolate the RAD52 gene product and to determine whether this gene might code for an essential function.

MATERIALS AND METHODS

Strains. The transformable yeast strain XS95-6C (MATα rad52-1 ura3-52 leu2-3 leu2-112 trp1 and his3-1) was used in most of the experiments. It was constructed by first crossing gl60/2d (MATα rad52-1 ade2-1 arg4 arg9 trp1 his5 leu1-1 ilv3 and leu2) from the Yeast Genetic Stock Center (Berkeley, CA.) to RH218 (MATa trp1-289) and then back crossing a resulting MATa rad52-1 trp1 derivative twice to DBY746 (MATα trp1-289 ura3-52 his3-1 leu2-3 leu2-112) (similar to SHY strains in ref. 2), kindly supplied by D. Botstein. The bacterial strains used were HB101 (CA600 leu⁻ B1⁻ thr⁻ pro⁻ lacz⁻ Sm⁻ recA⁻, rB⁻ mB⁻ SUII) supplied by R. Davis, JA300 (thr⁻ leuB6⁻ thi⁻ thyA⁻ trpC117⁻ hsrK⁻ hsmK⁻ strR) (29) supplied by John Carbon, and DB6507 (HB101 pyrF⁻) supplied by D. Botstein.

Plasmids. A bank of near random (Sau3A) partial fragments cloned into the BamH1 site of the vector YEp13 was kindly supplied by K. Nasmyth (16). YRp7 and YIp5 were obtained from D. Botstein.

Yeast transformation. Yeast transformation was performed using a modified version of the Hinnen, Hicks and Fink method (9), as suggested by V. MacKay and R. Hitzeman. 100ml of YEPD culture, grown to 2x10⁷ cells/ml, was collected and washed in water. The resulting pellet was resuspended in 5ml of S.E.D. (1M sorbitol, 25mM EDTA pH8.0 and 50mM dithiothreitol) and incubated 10 min. at 30°C. After a wash in 10ml 1M sorbitol, cells were resuspended in S.C.E. (1M sorbitol, 100mM Na citrate pH5 and 10mM EDTA) and 100 µl of zymolyase 60,000 (Kirin Brewery) at 1mg/ml was added. Cells were incubated at 30°C until 80-90% spheroplasting had occurred (1/2 to 1-1/2 hrs). Spheroplasting was monitored both microscopically and by loss of turbidity when spheroplasts were diluted into 10% SDS. Spheroplasts were washed 2 times in 1M sorbitol and once with CaS (1M sorbitol, 10mM CaCl₂ and 10mM Tris-HCl pH7.4). Spheroplasts were resuspended in 0.5ml CaS and divided into 100 µl aliquots. 5-20 µg plasmid DNA was added to cells and the mixture was incubated 15 min. at room temperature. 1ml of 20% polyethylene glycol (PEG-4,000, Sigma), 10mM CaCl₂ and 10mM Tris pH7.4 was added to each sample and incubated 15 min. at room temperature. After gently spinning the spheroplasts out of the PEG solution, they were

resuspended in 150 µl of 1M sorbitol, 33.5% YEPD and 50mM CaCl$_2$.   50 µl
aliquots were plated in yeast regeneration agar onto minimal dropout plates as
described previously (9).

   E. coli transformation and plasmid preparation.   E. coli transformation was
performed as described by R. Davis et al. (4).   Small scale plasmid prepara-
tions were performed by the method of Holmes and Quigley (11) and large scale
isolations plasmid were carried out using a protocol of Birnboim and Doly (1)
scaled up to 1 liter by S. Conrad.

   Restriction, ligation, S1 nuclease digestion and BAL31 digestion.   All of
the enzymes except BAL31 were obtained from Bethesda Research Laboratories and
the B.R.L. suggested procedures were followed.   BAL31 was obtained from New
England Bio Labs and their procedure was followed.

   X-ray source.   A Machlett OEG-60 X-ray source operated at 50 kV and 25 mA
was used.   The exposure rate was 240 R/sec.   The f factor for the exposure
conditions used was .90 rad/R.

RESULTS AND DISCUSSION

Cloning of RAD52

   A leu2 rad52-1 yeast strain (XS95-6C) was transformed with YEp13 (3)
containing near random yeast DNA inserts (16) and Leu$^+$ transformants were
selected.   Approximately 2,200 Leu$^+$ transformant colonies were picked to
master plates (50 per -leu plate) and grown up for 2 days at 30°C.   They were
then replica plated to YEPD plates and the replicas were treated with 50 krads
of X-ray.   Two transformants showed significant X-ray resistance and these were
tested further.   Survival curves on these transformants demonstrated that they
had near wild type survival after X-ray treatment (data not shown).   Cells
derived from these transformants which had lost their plasmid became simul-
taneously leucine auxotrophs and radiation sensitive.   Plasmid DNA was isolated
from the two Rad$^+$ transformants by transforming E. coli with a crude yeast
DNA preparation and selecting for ampicillin-resistant E. coli colonies.   From
these E. coli transformants, plasmid DNA was isolated and restriction analyses
carried out.   One plasmid contained a 5.9kb insert and the other one an insert
slightly over 20kb.   Preliminary restriction analysis of the two inserts was
consistent with the smaller insert being part of the larger insert.   Because
of complications associated with obtaining large amounts of plasmid DNA and a
detailed restriction map from the plasmid with the 20kb insert, we concen-
trated on the plasmid containing the smaller insert.   A restriction map of the
smaller plasmid is shown in Figure 1.   Reintroduction of either of the 'RAD52'
plasmids into XS95-6C (rad52-1 leu2) resulted in a Leu$^+$ Rad$^+$ phenotype for
all of the transformant colonies tested (100 for each plasmid). These results
are consistent with the plasmids containing either the RAD52 gene or a
suppressor of the rad52-1 mutation.   Although the rad52-1 mutation is not non-
sense suppressible. it is possible that another gene on a high copy number
plasmid might suppress the rad52-1 mutation or by-pass its defect.   Experiments
discussed below demonstrate that this is not the case and that at least the
smaller plasmid contains the RAD52 gene and not a suppressor.
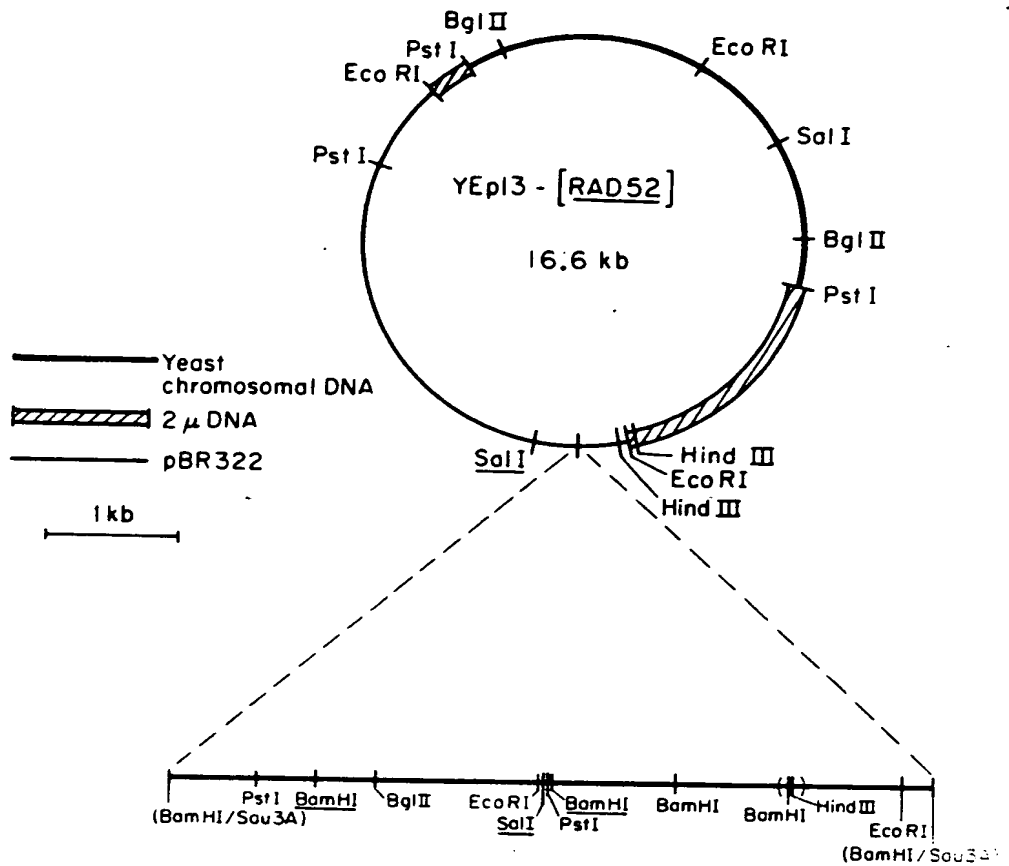
Figure 1.   Restriction map of YEp13-[RAD52].   This plasmid consists of a 3.3 kb yeast Sau3A fragment, containing the RAD52 gene, cloned into the BamHI site of the YEp13 vector (pBR322, the yeast LEU2 gene and the part of the yeast 2μ plasmid which includes the origin of replication) (3).

Subcloning of 'RAD52' and 'rad52' Fragments

From the original 5.9kb insert, we have subcloned several fragments into the YRp7 vector using standard techniques. A 3.3kb SalI fragment, consisting primarily of yeast DNA but with 275 base pairs from pBR322, and a 1.97kb yeast BamHl fragment were found to have 'RAD52' complementing activity (Figure 2). Unexpectedly, when this BamHl fragment was isolated in the opposite orientation in YRp7, it lacked activity. One trivial explanation for this difference was that the 1.97kb BamHl fragment in YRp7-C9 had been mutated or modified either by growth in E. coli or by the various biochemical manipulations during the subcloning procedures. We can rule this out because we have subcloned the 1.97kb fragment from YRp7-C9 back into YRp7 in the same orientation as YRp-C2-[RAD52] and this subclone now has RAD52 activity. The orientation difference we observe probably indicates that the activity of the BamHl fragment is dependent on part of the vector sequence, such as a promoter, or is being inhibited by part of the vector and that this dependence or inhibition is orientation specific. The BamHl-BglII subclones all lacked activity, indicating that the BglII site is probably either in the structural gene or between the gene and its regulatory region.

Integration Analysis of YRp7-A4 Sal-[RAD52]

In order to prove that we had cloned the RAD52 gene, rather than a suppressor of rad52-1, we examined integration events of one of the subclones. It has been demonstrated that integration of plasmids in yeast occurs via homologous recombination (8,17,26). Therefore, integration events at the chromosomal location of RAD52 would indicate that the plasmid contains the RAD52 region. Three independent spontaneous integration events were found in which the YRp7-A4-Sal-[RAD52] plasmid had integrated into the genome of XS95-6C. Two of these integration events were at the chromosomal location of TRP1 (data not shown) but one integrated at the chromosomal site of RAD52. The chromosomal location of this integrant was established by crossing it to a Rad$^+$ haploid of the opposite mating type and dissecting 10 asci from the resulting diploid (rad52[RAD52]/RAD52). All eight asci with four viable spore colonies yielded 4 Rad$^+$:0 rad$^-$ segregants and the two asci with three viable spore colonies yielded 3 Rad$^+$:0 rad$^-$ segregants. This segregation pattern establishes that the integration event occurred at or very close to the RAD52 chromosomal site on chromosome XIII. If the YRp7-A4-Sal-[RAD52] plasmid had not integrated at or very near the site of rad52-1 one would have expected some asci with 3 Rad$^+$:1 rad52-1 or 2 Rad$^+$: 2 rad52-1 segregations. The original integrant was also crossed to a rad52-1 lys7 trp1 strain and asci from this cross were dissected. The Rad$^+$ Trp$^+$ phenotypes segregated together as expected for an integrated plasmid containing both TRP1 and RAD52. Both were also linked to lys7 (P:NP:T = 4:0:4) and to a centromere (FDS:SDS = 5:2) at distances consistent with the map position of RAD52 (14).

The 1.9kb BamHl fragment has also been subcloned into YIp5, the URA3 integrating vector (2,28), and preliminary results indicate that this plasmid also integrates at the chromosomal location of RAD52.

Figure 2.   Subclones with and without RAD52 activity.   Various segments of the
original 5.9kb fragment subcloned into the SalI or BamHI site of the YRp7
vector (pBR322 with the yeast TRP1-ARS1 region).   The inserts in the
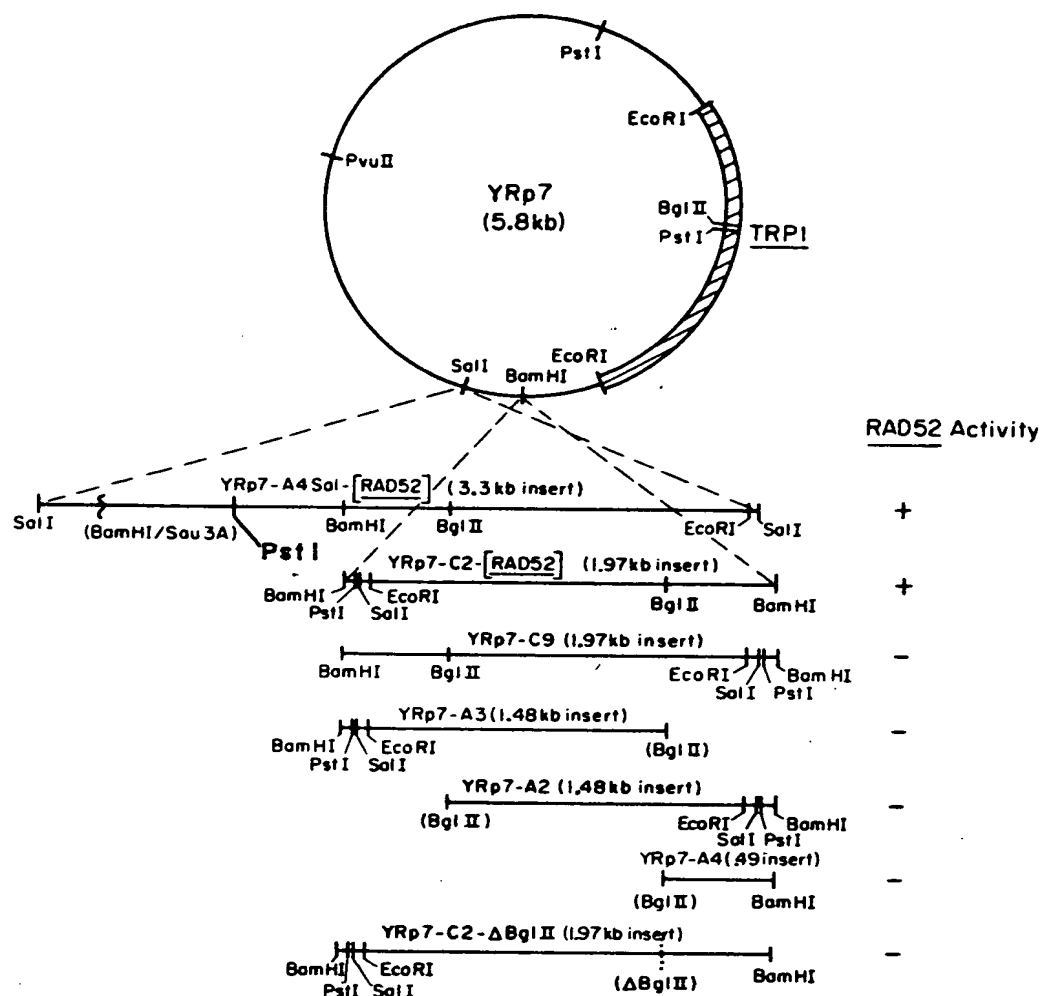YRp7-C2-[RAD52] and YRp7-C9 plasmids are identical except for orientation.

BAL31 Deletions

In order to more precisely define where the RAD52 gene is located within the 1.97kb BamHl fragment, we have isolated deletions into the insert from one end. Since the subcloning experiments (Figure 2) and the BglII deletion experiment (see below) indicated that the RAD52 region included the BglII site, we isolated deletions into the insert from the side furthest away from the BglII site (the left side in YRp7-C2-[RAD52], see Figure 3a). Because the isolation of BAL31 deletions is simplified by starting from a restriction site that is unique in the plasmid, we first deleted the short SalI fragment of YRp7-C2-[RAD52], using standard restriction and religation procedures. The resultant plasmid (YRp7-C2-ΔSal-[RAD52]) contains both unique SalI and BamHl sites (Figure 3a). This plasmid still complements the rad52-1 mutation, which is expected since the previously subcloned 3.3kb SalI fragment, which lacks the short yeast BamHl to SalI region, has RAD52 activity (Figure 2). The YRp7-C2-ΔSal-[RAD52] plasmid was linearized by restriction with SalI and digested with BAL31 for various lengths of time from 15 min. to 2 hr. Most of the time points gave large deletions; all of the deletions we used were from the 15 and 30 min. time points. Since BAL31 causes deletions in both directions from the SalI site, we restricted with PvuII before ligations so that we could later determine the length of the deletion within the RAD52 region. PvuII leaves blunt ends, but since BAL31 ends are frequently not blunt we increased the number of blunt ends by filling in some of the overhangs using the Klenow fragment of DNA PolI, prior to blunt end ligation. Following ligation, we transformed E. coli and used restriction analysis (PstI and BamHl double digest) on mini plasmid preparations to screen for plasmids with different sized deletions up to about 1.5kb. Large scale plasmid preparations were done on 24 plasmids and these plasmids were reintroduced into the rad52-1 yeast strain XS95-6C in order to score for RAD52 activity. The deletions which were reintroduced into yeast are diagrammed in Figure 3b. The size of the deletions are approximations since agarose gel electrophoresis does not give exact lengths, but each deletion diagrammed did appear to be slightly different in length. Some deletions appeared to be of the same size and these duplications are not listed in Figure 3b although the RAD52 activity results for these apparent repeats were consistent. Some of the sizes of the deletions might be overestimates if BAL31 had actually digested much further in the SalI to PvuII direction and had deleted past the PvuII site. This seems unlikely for most of the deletions since the distance from SalI to PvuII is ∿1.4kb and most of our deletions were 1kb or shorter. The results for two deletions (G1 and 02) are inconsistent with the results from the other deletions; although they appeared to be short deletions they lacked activity. These cases might represent longer deletions which appear shorter because of the addition of random short DNA fragments during ligation. The rest of the deletions are consistent with deletions of about 900 bases retaining RAD52 activity, while longer deletions inactivate the gene. Therefore, the RAD52 gene is at most 1kb in length.

Deletion of BglII site

A deletion-frameshift mutation in RAD52 has been isolated to determine whether RAD52 codes for an essential function; only two rad52 alleles exist (5) and neither is known to be nonsense suppressible. If both alleles are

A.

YRp7-C2-[RAD52]
(7.78 kb)

Deletion of
Sal I fragment

YRp7-C2-ΔSal-[RAD52]
(7.44 kb)

B.

BAL31 DELETIONS FROM SALI

	RAD52 Activity
Original 1.9 kb RAD52 fragment	
SalI .... BglII ... BamHI	
03:50 bp Δ —	+
R3:440bp Δ ————————	+
B2:590bp Δ ———————————	+
W2:640bp Δ ———————————	+
W3:690bp Δ ————————————	+
Y4:740bp Δ —————————————	+
W4:890bp Δ ——————————————	+
G3:910bp Δ ——————————————	—
B1:920bp Δ ——————————————	—
B3:930bp Δ ——————————————	—
R2:940bp Δ ——————————————	—
R1:980bp Δ ———————————————	—
Y2:1240bp Δ ————————————————————	—
Y3:1370bp Δ —————————————————————	—
G1: 410 bp Δ —————————	—
02:630bp Δ —————————	—
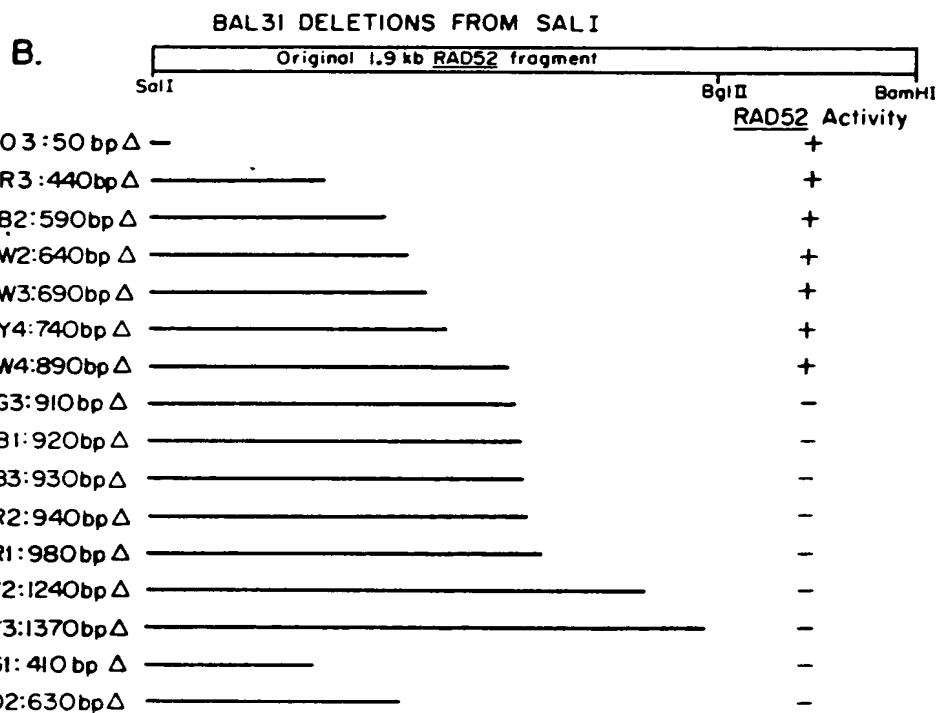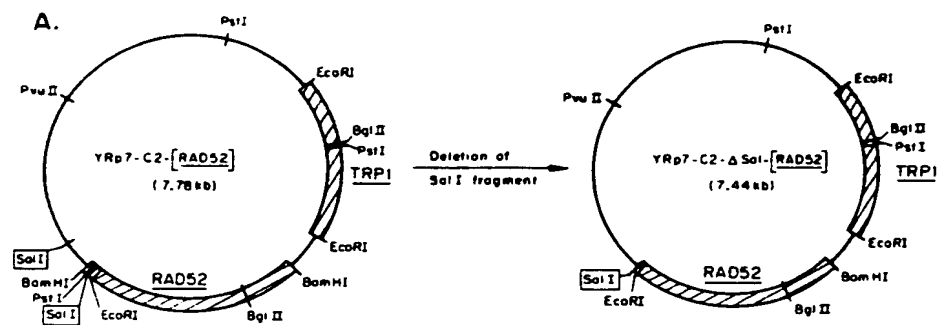
Figure 3. BAL31 deletions. A. Removal of short SalI fragment from
YRp7-C2-[RAD52] before BAL31 deletions isolated.   B. BAL 31 deletions from
SalI site into RAD52 region. Line represents length of deleted segment.

leaky, then it is possible that RAD52 might encode an essential function. The
rad52-2 allele is clearly leaky, but it is not known if rad52-1 is also leaky
(5,27). The rad54-3 mutation is more X-ray sensitive than rad52-1 (J. Game,
personal communication) and since rad52 and rad54 are in the same epistasis
group, it might indicate that rad52-1 is a slightly leaky allele. It is known
that diploids homozygous for rad52-1 have extremely low (~10-20%) plating
efficiency and also spontaneously undergo chromosome loss (15) indicating a
probable role for this gene during normal cell division.

Since the previously discussed subcloning experiments indicated that the
BglII site in the 1.97kb BamH1 fragment was probably in the RAD52 gene, we
decided to isolate a "null" allele of rad52 by constructing a four base pair
deletion of the BglII site using SI nuclease. The 1.97kb BamH1 fragment was
first subcloned into pBR322 so that BglII site in the BamH1 fragment was the
only BglII site in the plasmid. This plasmid was restricted with BglII,
treated with S1 single-strand exonuclease, blunt-end ligated, recut with BglII
(to decrease transformation ability of plasmids still containing a BglII site),
and transformed into HB101. Mini plasmid preparations on the transformants
showed that the BglII site was missing from plasmids in most of the transform-
ants. Following large scale preparations of two plasmids with independently
derived deletions of BglII, we subcloned these deletions into both YRp7 and
YIp5 in the proper orientation for RAD52 activity. When the deletions in YRp7
were introduced into yeast they failed to complement rad52-1 (Figure 2). This
demonstrates that the BglII site is either in the structural gene or in a
region essential for transcription or translation. We have recently integrated
these deletions in YIp5 into a wild type strain of yeast and are currently
examining excision events in both haploids and diploids in order to determine
whether RAD52 codes for an essential function. If these experiments show that
RAD52 codes for a nonessential function, our BglII deletion allele will still
be valuable as a nonleaky allele.

NOTE ADDED IN PROOF

Kenji Adzuma (personal communication), in the laboratory of Dr. H. Ogawa
(Osaka University, Osaka, Japan), has independently isolated a 1.98kb BamH1
fragment which complements the rad52-1 mutation. This fragment appears to be
identical to our 1.97kb BamH1 fragment, since both share a common restriction
map.

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17. CLONING OF A DNA REPAIR GENE IN YEAST*

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A fragment of DNA which restores resistance to UV, gamma rays, and methyl methanesulfonate in both rad6-1 and rad6-3 mutants has been isolated on a recombinant plasmid, YEp13. Recombinant plasmids containing such DNA segments were obtained by transforming a leu2-3 leu2-112 rad6-1 strain to LEU+ and screening for UV resistance among the LEU+ transformants. Three classes of recombinant plasmids, based on restriction with BamHI, were obtained. The cloned DNA segment complementing rad6 was transferred to an integrating plasmid containing the yeast URA3 gene, and then used to determine the site of recombination of the cloned DNA. Preliminary genetic experiments suggest that the cloned segment integrates at the rad6 locus. Subcloning of this segment has yielded a 1.9 kb fragment which still functions in complementation of rad6. When this fragment is used as a probe for hybridization to total yeast RNA, two transcripts are observed.

INTRODUCTION

Procaryotic and eucaryotic organisms possess many different mechanisms which enable them to repair damage induced in their DNA by a wide variety of physical and chemical agents. Modification of the DNA damage, however, depends on the genetic constitution of the organism as well as the post-exposure conditions. In Escherichia coli, repair of ultraviolet light (UV) induced damage is governed by genes in two epistasis groups - one controlled by the uvrA+, uvrB+, uvrC+ system involved in excision of pyrimidine dimers, and the other controlled by the recA+-lexA+ system (10,51), involved in postreplication repair. The sensitivity of double mutants consisting of uvrA and recA is such that one pyrimidine dimer is sufficient to kill the cell, indicating that no other pathways play a major role in repair of UV damage in E. coli (14).

Many cellular repair processes, such as excision repair and postreplication repair, also occur in eucaryotes. However, relatively few DNA repair mutants are known in higher eucaryotes. In the yeast

Saccharomyces cerevisiae, which has a well-characterized genetic system, a large number of radiation sensitive (rad) mutants representing over 30 distinct genetic loci have been isolated and many have been characterized extensively in terms of their effect on UV induced mutations (19,21,39), chemically induced mutations (31,33), and their repair defects. Comparisons of sensitivities to killing of various double mutant combinations indicate that three epistasis groups exist for repairing UV induced DNA damage (6, see 11 for review). One group consists of nine genes, RAD1, RAD2, RAD3, RAD4, RAD7, RAD10, RAD14, RAD16, and MMS19, involved in excision of UV-induced pyrimidine dimers (32,36,42,43,48,49). Like the excision defective uvrA and uvrB mutants of E. coli, the yeast excision defective mutants show enhanced frequencies of mutations following UV irradiation. Experiments with double mutants consisting of the rad mutants defective in excision coupled with cdc9, which is temperature sensitive for growth and lacks detectable DNA ligase activity in vitro (18), suggest that rad1, rad2, rad3, rad4, and rad10 mutants are defective in an initial incision step required for pyrimidine dimer removal and rad14 is defective in a step subsequent to incision (50). In addition, it has been shown that cell-free extracts from rad1, rad2, rad3, rad4, and rad10 strains are capable of removing dimers from preincised UV irradiated DNA (44). However, it has not been possible to demonstrate pyrimidine dimer incising activity in cell-free extracts of yeast.

The RAD52 epistasis group consists of eight genes (RAD50 to RAD57) which confer sensitivity primarily to ionizing radiation (8), while UV sensitivity is enhanced mainly in the presence of a defect in excision (6). Mutability induced by UV or gamma rays in mutants of genes of the RAD52 group is similar to that observed in RAD+ strains (19,25). Many of the mutants in this group, particularly rad52, affect recombination and DNA strand break repair. The rad52-1 mutant is defective in spontaneous, UV and ionizing radiation induced homologous mitotic recombination (23,38,40), mitotic gene conversion (17), meiotic recombination between homologous chromosomes (9,38), UV induced sister chromatid recombination (37), and in mating type interconversion (23) which occurs by transposition of DNA copies from silent a and α loci to the mating type locus. The rad52-1 mutation also blocks chromosomal integration of gapped-linear or linear molecules but not of circular molecules (30). In addition, both the rad51-1 and rad52-1 mutants are defective in the repair of DNA double strand breaks (16,41). The rad52-1 mutants are also not as proficient as wild type in postreplication repair of UV damaged DNA (34).

Mutants of genes in the third epistasis group affect sensitivity to both UV and ionizing radiation and consist of rad6, rad8, rad9, rad18, rev1, rev2, rev3, and mms3. Many of these mutants show reduced UV mutability for at least some, if not all, loci tested (19,22,24). In addition, some of them have pleiotropic effects on spontaneous mitotic gene conversion, spontaneous mutation, and DNA repair (see 11 for review). The rad18-3 mutant increases rates of spontaneous mitotic recombination (1). Both alleles of the rad6 locus, rad6-1 and rad6-3, enhance spontaneous and UV induced mitotic heteroallelic recombination in diploids and are proficient in spontaneous and UV induced unequal sister chromatid recombination in haploids (26). Both rad6 mutants show no induced mutations with UV,

ionizing radiation (19,25), ethyl methanesulfonate,
N-methyl-N'-nitro-nitrosoguanidine, and a wide variety of other chemical
mutagens (31). The rad6-1 and rad18-2 mutants show a great inhibition of
postreplication repair of UV damaged DNA while the rev3-1 mutation does not
affect it (34).

Our aim has been to study the genes and gene products involved in DNA
repair and mutagenesis in order to elucidate the molecular mechanisms of
these important cellular responses. We have begun this study by cloning one
of the genes, RAD6, which probably plays an important role in the regulation
of these two cellular processes and report here our preliminary results.

MATERIALS AND METHODS

Strains: The yeast strains LP2530-2A: MATa leu2-3 leu2-112 his5-2
trp1-1 rad6-1 and LP2652-9C: MATa leu2-3 leu2-112 trp5-c ura3-52 rad6-3
were constructed by standard techniques of yeast genetics and used for
transformation. Strain AB320 HO met4-1 ade2-1 lys2-1 trp5-2 leu2-1 can1-100
ura3-1 and/or ural was obtained from J. Hopper and S150-2B MATa leu2-3
leu2-112 ura3-52 trp1-289 his3Δ1 from S. Baim. Escherichia coli strain
HB101 was obtained from H. Eberle.

Plasmids used for cloning: YEp13 (2), a 10.7 kb hybrid of pBR322
containing a yeast DNA fragment with the 2 μ circle origin of replication,
enabling it to replicate autonomously in yeast, the yeast LEU2 gene, which
complements E. coli leuB, the col E1 origin of replication, and the genes
conferring ampicillin (amp^R) and tetracycline resistance (tet^R). A
single BamHI site, used as the cloning site, is present in the tet gene;
insertion in the BamHI site inactivates the tet gene.

YIp5 (45) a 5.5 kb plasmid, also derived from pBR322, replicates
autonomously in E. coli but not yeast, contains the amp and tet genes, and
the yeast URA3 gene.

pAB108, a 6.1 kb derivative of pBR322, contains the amp gene and
replicates autonomously in both E. coli and yeast because of the yeast
chromosomal replicator ARS2, and contains the URA3 gene for selection in
yeast. pAB108 contains unique EcoRI, HindIII, BamHI sites, was constructed
by and obtained from S. Baim. pBEU49 is a recAo281 multicopy plasmid
obtained from J. Clark and has a BamHI fragment containing the recA gene of
E. coli.

DNA purifications: Plasmid DNA from yeast was purified by a modified
method of Hirt (13). Yeast DNA was purified by a mini-prep method obtained
from G.S. Roeder (unpublished results). Growth of E. coli containing
plasmids was carried out as described (29) but with nucleosides omitted.
Plasmid DNA was then purified by the cleared lysate method of Clewell and
Helinski (5) but with 0.1 percent Triton X-100 instead of Brij 58. For
screening of large number of E. coli containing plasmids, the procedure of
Ish-Horowicz and Burke (15) for DNA extraction from plasmid or cosmid
cultures was used.

Pool DNA for transformation: The pool of yeast DNA sequences used for transformation, and designated pool 35, was constructed by K. Nasmyth and S. Reed and obtained from J. Hopper. Genomic DNA was purified from strain AB320, digested partially with Sau3A, and fragments of average size of 10 kb purified by sucrose gradients. These fragments were ligated into the BamHI site of YEp13 and used to transform E. coli to amp^R. Transformation of yeast was carried out as previously described (12).

Scoring of UV, X-ray and methyl methanesulfonate sensitivity: Methods were as described by Prakash and Prakash (35).

Gel electrophoresis, transfer to nitrocellulose filters, preparation of probes and hybridization: Restriction endonucleases were purchased from New England Biolabs and used in 0.09 M Tris-HCl, pH 7.4 - 0.01 M MgCl$_2$ buffer. Electrophoresis in agarose slab gels (Model 800, purchased from Aquebogue Machine Shop, Aquebogue L.I., N.Y.) to separate restriction fragments was carried out in 89 mM Tris-HCl, 23 mM phosphoric acid, 2.5 mM EDTA buffer, pH 8.3. Agarose gels also contained 0.5 µg/ml ethidium bromide. The restriction fragments were photographed under short-wave ultraviolet light with Polaroid type 57 film. DNA fragments to be used as probes were isolated by electroelution from agarose gels as described by Zaret and Sherman (52). Nick translation of fragments for radioactive labeling was carried out by using the nick translation kit purchased from Amersham. The specific activities of various probes ranged from 1.4 x 10^7 to 6.1 x 10^7 cpm/µg DNA and total of about 2 x 10^6 cpm was used for each Southern blot. The method of Southern (46) was used to transfer restriction fragments to nitrocellulose paper and hybridizations were carried out as described by Zaret and Sherman (52).

RESULTS AND DISCUSSION

Isolation of RAD6-containing plasmids: We cloned the RAD6 gene by screening for UV resistant colonies in a population of transformed rad6-1 mutants cells. This method of complementation has been used successfully to clone various other yeast genes, such as cdc10, cdc28, and rad52 (4,28 and Schild and Mortimer, personal communication). However, before making use of this procedure, several precautions had to be taken because of the nature of the only existing rad6 alleles. Both rad6-1, isolated by Cox and Parry (7), and rad6-3, isolated in our laboratory (35), are nonsense alleles suppressed by both amber and ochre suppressors (20,47). Therefore, we included an amber allele, trp1-1, and an ochre allele his5-2, in the rad6-1 strain to be transformed for isolation of the RAD6 gene. Any putative RAD6-containing clones could then be tested for the presence of a suppressor activity. The restoration of the RAD+ phenotype to a rad6 mutant could arise from either the presence of the RAD6 gene on the cloned DNA fragment, or the presence of any other gene which could suppress the rad6 phenotype. Any strains which were RAD+ as a result of cloning of a translational suppressor could be eliminated by testing RAD+ clones for ability to grow without histidine or tryptophan. A second precaution taken was to determine that strain AB320, from which pool 35 DNA had been constructed, did not contain any genes that might suppress either rad6-1 or rad6-3. This was done by crossing AB320 to rad6-1 and rad6-3 suitably marked haploids and analyzing the segregation

pattern for the rad6 phenotype in the resulting progeny. In both crosses, rad6-1 and rad6-3 segregated 2:2, indicating that strain AB320 did not contain any suppressors of either rad6-1 or rad6-3.

Strain LP2530-2A, MATa leu2-3 leu2-112 his5-2 trp1-1 rad6-1, was transformed to LEU+ with pool 35 DNA. LEU+ transformants were then transferred to media lacking leucine and tested for their response to UV irradiation as well as for growth in the absence of histidine or tryptophan. Nine independent LEU+ clones were obtained which were UV resistant and retained dependence on histidine and tryptophan for growth. However, pool 35 contains a suppressor present in about 1 percent of LEU+ transformants which suppresses trp1-1 poorly, does not act on the amber allele ade5-7, or the ochre alleles lys1-1, ilv1-1, and can1-100, but suppresses trp1-289, aro1B, and met8-1 very well (R. Mortimer, personal communication). In order to eliminate the possibility that the putative RAD6 clones contained this suppressor which was also suppressing rad6-1 and rad6-3, we transformed strain S150-2B to LEU+ with each plasmid and tested 30 LEU+ transformants from each experiment. None of the LEU+ transformants were TRP+, indicating that the putative RAD6 clones did not contain suppressors of trp1-289.

Plasmid DNA obtained from each of the nine clones was used to transform E. coli strain HB101 to ampicillin resistance. Plasmid DNA from each of the nine transformed strains was then purified by by amplification with chloramphenicol and centrifugation through CsCl. The purified DNA was used to transform LP2530-2A (rad6-1) and LP2652-9C (rad6-3) to LEU+. All LEU+ transformants were UV resistant and still required tryptophan and histidine for growth. In addition, resistance to the lethal effects of X-rays and methyl methanesulfonate was obtained and sporulation ability was restored in diploids obtained by crossing rad6-1 haploids to transformed rad6-1 haploids of opposite mating type. These results indicate that the nine plasmids, designated pTB12 through pTB20, all contain a DNA fragment which restores RAD6 function to rad6-1 and rad6-3 strains.

Restriction maps of putative RAD6-containing plasmids: Digestion with BamHI was used to determine whether any similarities existed among the nine yeast DNA inserts. Three groups were obtained, based on the types of fragments obtained following digestion with BamHI: (a) one insert fragment in pTB13, pTB14, pTB15, pTB16, and pTB20 (b) two insert fragments in pTB17 (c) and no insert fragment in pTB13, pTB18, and pTB19. The extent of homology among the cloned yeast DNA fragments was determined by mapping several restriction sites in pTB16, one of five plasmids in class (a), and pTB17, the only plasmid in class (b). The results are presented in Figure 1. The 5.2 kb BamHI fragment of pTB17 is homologous to the 5.2 kb BamHI fragment of pTB16 and both fragments contain the RAD6 function since transfer of each fragment in both orientations into the BamHI site of YEp13 resulted in plasmids capable of transforming rad6-1 and rad6-3 strains to RAD+ with high efficiency. Restriction maps for members of class c have not yet been completed, however, hybridization results indicate that this class contains a small deletion within the 4.2 kb HindIII fragment present in both pTB16 and pTB17 (Figure 2). For this experiment, the 5.2 kb BamHI fragment from pTB17 was used as a probe to hybridize to HindIII or BamHI digested
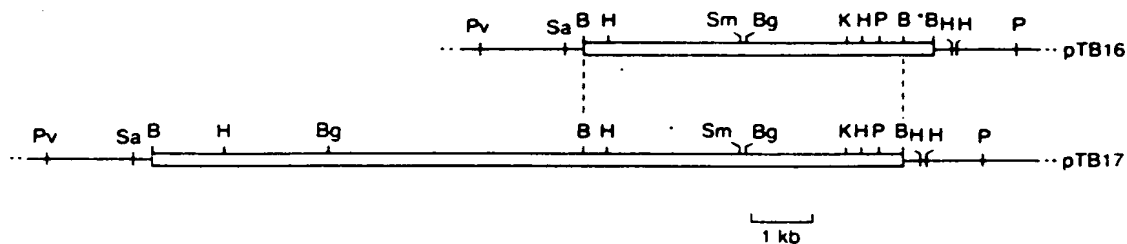
Figure 1.   Restriction maps of recombinant plasmids containing the RAD6 gene.

Insert of yeast DNA segments, given by open bars, is in the BamHI site of the tet gene of YEp13. The thin line represents YEp13 DNA. The extent of overlap and orientation was determined by analysis of restriction sites and Southern gels. Symbols for restriction enzymes are as follows: B, BamHI; *B, BamHI/Sau3A junction; Bg, BglII; H, HindIII; K, KpnI; P, PstI; Pv, PvuII; Sa, SalI. The 5.2 kb BamHI fragment of pTB16 is homologous to the 5.2 kb BamHI fragment of pTB17, which contains an additional 6.6 kb BamHI fragment, as shown. In pTB16, the insertion of the Sau3A fragment occurred such that the left end, near the SalI site, generated a BamHI site, while the other end, near the HindIII site (on the right hand in the figure), generated a site not cut by BamHI and is designated *B. However, an additional BamHI site occurs about 0.5 kb to the left of *B and BamHI digestion of pTB16 yields an insert fragment of about 5.2 kb.

Figure 2.   Structural homologies among RAD6 plasmids.

The 5.2 kb BamHI fragment of pTB17 was purified by electroelution and labeled by nick translation. Digested plasmid DNA samples were separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with the ^{32}P labeled probe. Lane a: plasmid DNA from pTB60, which contains the 4.2 kb HindIII fragment of pTB16 cloned into pAB108 (see below), digested with HindIII; lanes b, f, j, and k, plasmid DNA from pTB13, pTB13, pTB18 and pTB19, respectively, digested with HindIII; lanes c, d, e, and i: plasmid DNA from pTB17, pTB16, pTB16 and pTB14, respectively, digested with BamHI. Lane d contains about 1/4 the DNA present in lane e. Lanes g and h contain HindIII digested YEp13 and BamHI digested pBEU49 DNA, respectively. Lane l contains the purified HindIII fragment obtained from pTB60. The left hand side of the gel is somewhat skewed upward, giving those fragments an apparently larger size than they actually have, as observed in many other gels.

plasmids from all three classes. Lanes b, f, j, and k contain plasmids of class c, and the probe hybridizes to only one fragment of HindIII digested DNA in each case, and the size of that fragment is 3.5 kb. Lanes c, d, e, and i, on the other hand, contain BamHI digested plasmids of class a, and in these cases, the fragment which hybridizes with the 5.2 kb BamHI probe is about 5.2 kb. Lane g, which contains HindIII digested YEp13 (the vector without any insert) and lane h, which contains BamHI digested pBEU49 DNA, a plasmid containing the E. coli recA+ gene on a BamHI fragment, do not hybridize with the probe, as expected, since there is no homology between the yeast DNA probe with either vector alone or with vector containing E. coli DNA. Identical patterns of hybridization to that observed in Figure 2 are obtained if the 5.2 kb BamHI fragment or the 4.2 kb HindIII fragment of pTB16 are used as probes.

Confirmation that cloned fragments contain RAD6 DNA: Since complementation is not sufficient proof for the identity of a cloned gene, that RAD6 was contained in the plasmids described above was verified by mapping the cloned fragment to the yeast chromosome . The 5.2 kb BamHI fragment of pTB16 was purified and ligated into the single BamHI site of YIp5. This plasmid, designated pTB33, was used to transform LP2652-9C (rad6-3 ura3-52 trp5-c) to URA+. Since YIp5 does not replicate autonomously but transforms yeast by integration, and since the ura3-52 allele does not recombine and is thought to be a small deletion (45), integration of the URA3 gene should occur. in the region of the yeast genome which shows homology with the cloned yeast DNA fragment. The URA+ transformants were tested for sensitivity to UV irradiation: six were UV resistant and three UV sensitive; the latter three might have arisen as a result of gene conversion. One of the rad6-3 URA3+ trp5-c integrants was crossed to a RAD+ ura3-52 TRP5+ strain. The URA3 gene is located on chromosome V, whereas the TRP5 gene is on chromosome VII, about 20 cM away from RAD6 (27). Analysis of 20 tetrads obtained after sporulation of diploids yielded 14 parental ditype and 6 tetratype tetrads, giving 15 percent recombination. The linkage of ura3 to trp5 indicates that ura3 had integrated at the rad6 locus and that the RAD6 gene was present on the plasmid pTB33.

Integration at the RAD6 locus would generate a fragment of about 10.5 kb following digestion of total yeast genomic DNA with BglII, as indicated in Figure 3, whereas integration elsewhere in the genome would yield a BglII fragment whose size cannot be predicted since the relation of the flanking BglII sites to the BglII site in the integrated segment is not known. Further evidence for the identity of the cloned fragment as the RAD6 gene was obtained by digesting genomic DNA obtained from two RAD+ integrants and three rad6 integants with BglII and hybridizing to a pBR322 probe (Figure 4). As predicted for integration at the RAD6 locus, the size of the BglII fragment hybridizing to the probe is about 10.5 kb. Also as expected, vector sequences are present in the genomic DNA of all integrants (lanes a to e) whereas no hybridization was observed with genomic DNA prepared from two non-transformed strains (lanes f and g).

Subcloning and preliminary characterization of the RAD6 gene: In order to identify the smallest unit having RAD6 function, it was necessary to obtain smaller fragments of insert DNA. The HindIII fragment present within the two BamHI sites of pTB16 was cloned into the single HindIII site of
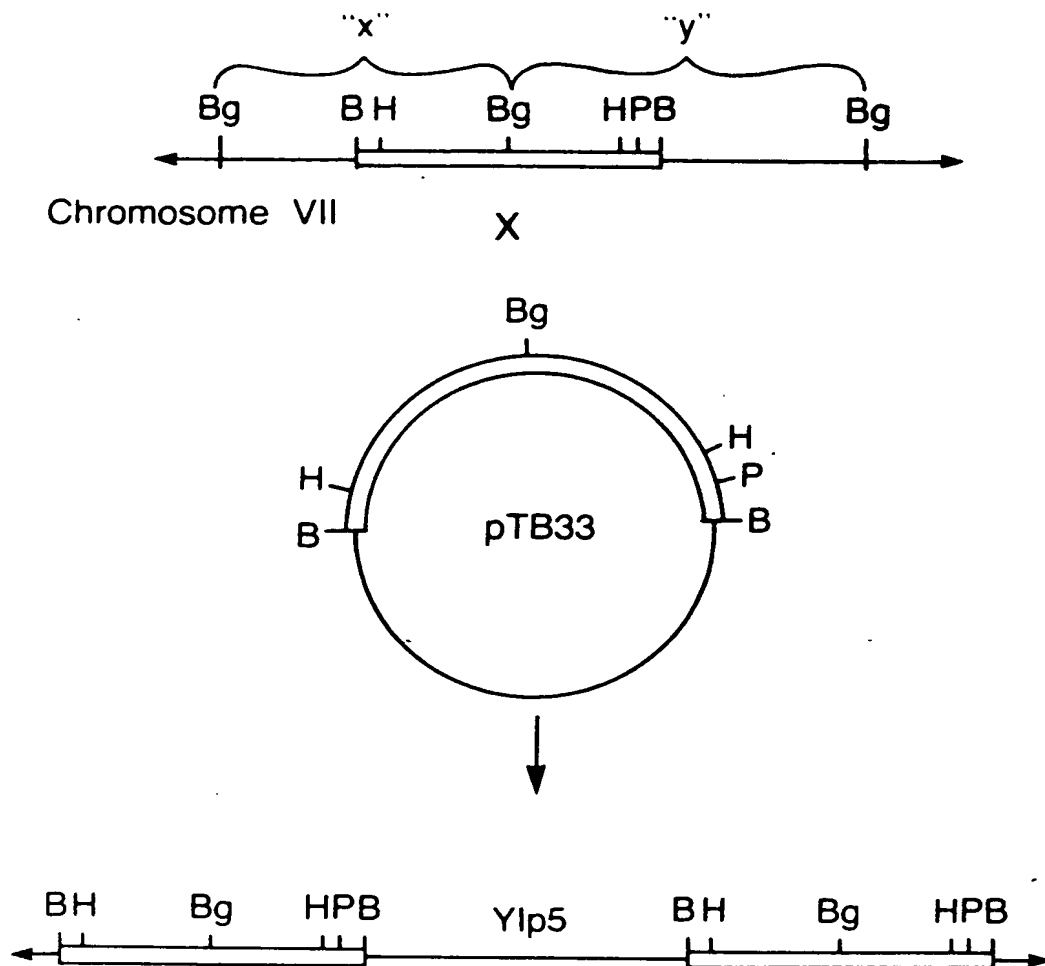
Figure 3.   Integration of a DNA segment on a plasmid by recombination with
its region of homology in the genome of yeast.

Plasmid pTB33 was constructed by insertion of the BamHI fragment of pTB16
into the BamHI site of YIp5. The structure of the resulting plasmid has not
yet been confirmed but is assumed to be as given above. The open bar refers
to the cloned segment on pTB33 and its corresponding region of homology on
chromosome VII, the location of the RAD6 gene. "X" and "Y" refer to the
distance between the BglII site in the cloned segment and the next BglII
site on chromosome VII, going leftward and rightward, respectively. The
thin line respresents chromosomal DNA. The lower linear chromosome
represents the integration of the cloned DNA segment into chromosome VII
with two copies flanking the vector sequence of YIp5. The predicted size of
the fragment generated by BglII digestion of chromosomal DNA from integrants
is about 10.5 kb. Symbols for restriction enzymes are as given in Figure 1.
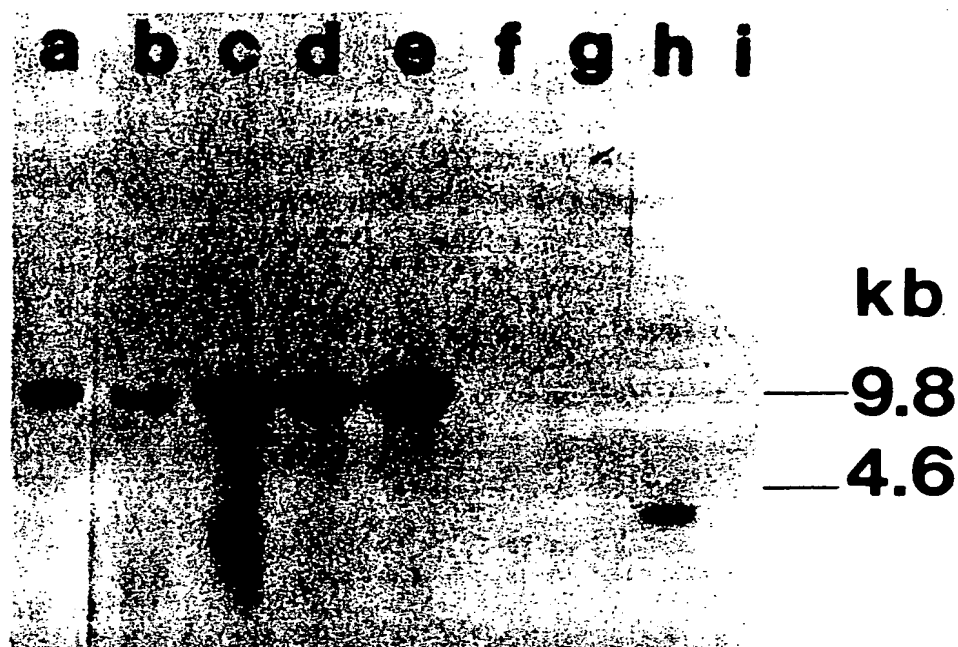
Figure 4.   Detection of pBR322 sequences in RAD+ URA+ integrants.

Chromosomal DNA from two UV resistant integrants, BES-2 and BES-5, lanes a
and b, respectively; three UV sensitive integrants, BES-7, BES-8, BES-9,
lanes c, d, and e, respectively, and two RAD+ non-transformed strains,
AB320, lane f, and #364, lane g, were digested with BglII and fragments
separated by electrophoresis on agarose gels. The DNA fragments were
transferred to nitrocellulose and hybridized to ^{32}P-labeled pBR322 DNA.
Lane h contains pBR322 DNA and lane i contains lambda DNA digested with
HindIII.

pAB108, generating the plasmid pTB60 (Figure 5).   pTB60 DNA was used to transform LP2652-9C to URA+ and all URA+ transformants obtained were also found to be UV resistant, indicating that the RAD6 gene was contained in the HindIII fragment. This 4.2 kb HindIII fragment, when hybridized to HindIII digested genomic yeast DNA, hybridizes to only one band of about 4.2 kb, indicating that no repeated sequences corresponding to this fragment are present in the yeast genome (Figure 6). Further subcloning of the RAD6 gene was achieved by deleting the rightward BglII/HindIII fragment of pTB60 by digestion with both BglII and BamHI (Figure 5). The resulting products were ligated and the mix used to transform HB101 to ampicillin resistance. Ampicillin resistant colonies were screened for the presence of the appropriate deletion. The plasmid generated in this way, pTB64, was also shown to contain RAD6 function as determined by the ability to restore UV resistance to the rad6-3 strain, LP2652-9C, indicating that this leftward HindIII/BglII fragment contains the RAD6 gene. Experiments are in progess to determine whether further subcloning of the RAD6 gene is possible while still retaining function.

As a prerequisite for identification of the RAD6 protein product by in vitro translation, we will identify the RAD6 transcript. In preliminary experiments, the 4.2 kb HindIII fragment of pTB60 was purified and used as a probe to hybridize to total yeast RNA. Three transcripts of about 1.7, 1.1, and 0.8 kb, were observed. The HindIII/BglII fragment present in pTB64 was isolated from HindIII/BglII double digestion of pTB60 by electroelution, since in the creation of pTB64, the BglII/BamHI ligation generates a sequence not recognized by either restriction enzyme. When this 1.9 kb HindIII/BglII fragment is used as a probe for hybridization to total yeast RNA, the largest transcript disappears and the two smaller ones remain. The possibility remains that further subcloning of the 1.9 kb HindIII/BglII fragment will yield an even smaller DNA segment which has RAD6 function and that when this smaller fragment is used as a probe for hybridization to yeast RNA, one of the two transcripts will not be seen. However, it is also possible that the RAD6 gene specifies two mRNAs, as has been found for the SUC2 gene, which specifies invertase (3); one transcript encodes the secreted, glycosylated form of invertase while the other encodes the intracellular form of invertase. In the case of the RAD6 gene, one transcript may be inducible, in response to DNA damaging agents, and the other may be constitutive.
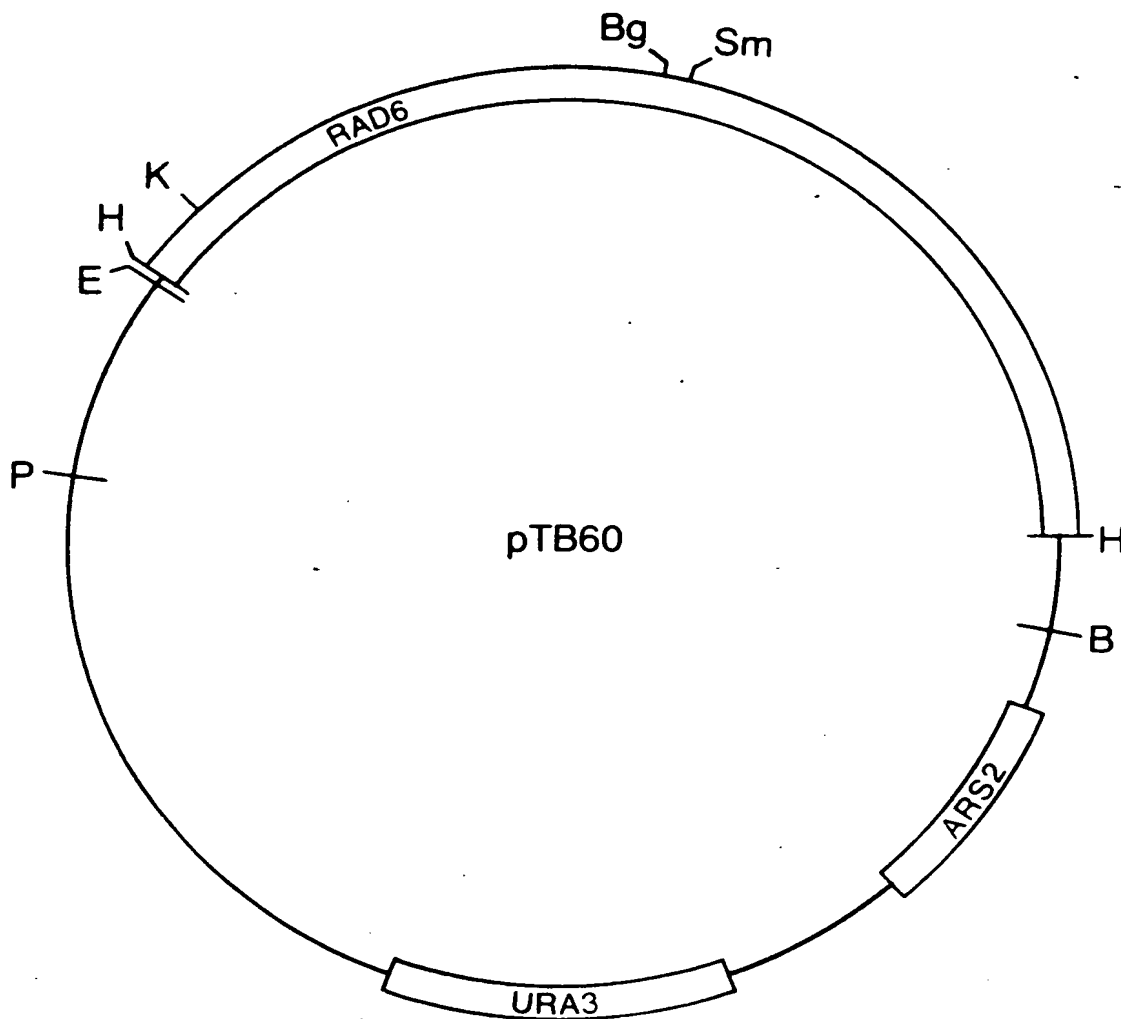
Figure 5.   Restriction map of pTB60.

The HindIII fragment from pTB16 was cloned into the single HindIII site of
pAB108, a 5.1 kb derivative of pBR322, and designated here by the thin line
and the two open bars, one with the URA3 gene and one with the ARS2 sequence
of yeast DNA.   The open bar marked RAD6 represents the HindIII fragment of
pTB16.   Symbols for restriction enzymes are as given in Figure 1.

a b c d e f g h i

—**4.2 kb**

H
3
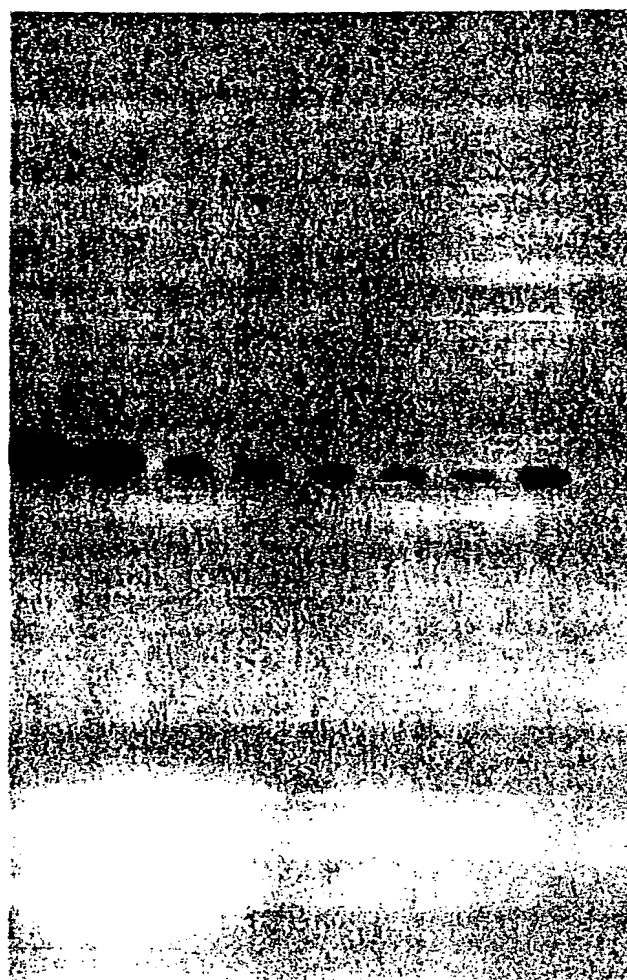
Figure 6.   Autoradiogram of Southern blot of total yeast DNA hybridized with
the 4.2 kb HindIII fragment of pTB60 labeled with ^{32}P.

Total DNA purified from strains BES-2, a RAD+ integrant, lane a; BES-7, a
rad integrant, lane b; RAD+ non-transformed strains: AB320, lane c, A364A,
lane d, #264, lane e, B-635, lane f, LP1777-9A, lane h. and a rad6-3
non-transformed strain MD-65, lane g, all digested with HindIII. Lane i
contains a HindIII digest of lambda DNA. DNAs were separated by agarose gel
electrophoresis, transferred to nitrocellulose, and hybridized to
^{32}P-labelled probe.

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18. MOLECULAR MECHANISMS OF DNA RECOMBINATION: TESTING MITOTIC AND MEIOTIC MODELS*

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A hyperhaploid n + 1 strain of Saccharomyces cerevisiae (LBL1) disomic for chromosome VII was employed to isolate hyper-rec and hypo-rec mutations affecting spontaneous mitotic gene conversion and intergenic recombination. The genotype of LBL1 permits simultaneous and independent identification of rec mutations that enhance or diminish gene conversion and those that enhance or diminish intergenic recombination. Five phenotypic groups of rec mutants were isolated following ultraviolet light mutagenesis. Rec mutations that simultaneously abolish or enhance both classes of recombinational events were detected. These results demonstrate that gene conversion and intergenic recombination are under joint genetic control in mitotic cells. Conversion-specific and intergenic recombination-specific rec mutants were also recovered. Their properties indicate that conversion and intergenic recombination are separable phenomena dependent upon discrete REC genes. The rec mutants isolated in LBL1 provide a method to test molecular models of mitotic and meiotic recombination.

INTRODUCTION

Interest in the mechanisms of mitotic and meiotic recombination in Saccharomyces cerevisiae has been heightened by recent demonstrations that genetic recombination plays a significant role in the regulation of gene expression in yeast and other eukaryotes (4,10). The mechanisms and pathways of yeast mitotic and meiotic recombination are exceptionally amenable to study. Molecular studies of DNA recombination and detailed genetic analyses suggest the existence of a variety of recombinational intermediates and modes of resolution (6). While several rec mutants have been isolated in yeast the extant collection does not appear to include variants defective in each step of recombination (1,6). We constructed an n + 1 hyperhaploid strain disomic for chromosome VII (5) for the isolation of a broad spectrum of rec mutations. The genotype of LBL1 permits detection of rec mutations affecting the initiation of recombination, establishment of Holliday structures or related intermediates, isomerization of Holliday structures and resolution of Holliday structures by endonucleolytic scission (11) or DNA replication (3). In this report we discuss the phenotypes of recently isolated rec mutants and analytic methods for identification of their putative defects. The latter methods were developed to characterize the recombinational phenotype of the spoll-1 mutant, as described in the accompanying article by Bruschi and Esposito.

MATERIALS AND METHODS

Yeast strains. The genotype of LBL1, the n + 1 hyperhaploid disomic for chromosome VII, is given in Figure 1.

Media and techniques. The recipes for media have been reported previously (8). Strain LBL1 was maintained on slants of leucineless tryptophanless omission medium or frozen in 50% (v/v) glycerol at -70°C. Before each experiment single colony isolates of LBL1 were obtained by streaking on leucineless trytophanless omission medium or synthetic complete medium followed by incubation at 24°C. The genotypes of putative ade5, ade6, and ade5 ade6 segregants of LBL1 were verified by complementation analysis employing ade5 and ade6 test strains. Segregation of cly8, a heat sensitive lethal mutation, was monitored by failure of growth on glucose nutrient medium at 36°C.

Determination of mitotic rates of restitution of haploidy, intergenic recombination and gene conversion. The rate of occurrence of each type of mitotic event was determined by the method of the median of Lea and Coulson (12) as previously reported (5,9,13). The LBL1 strain was plated on synthetic complete medium and incubated at 24°C. Twenty-five colonies having a diameter of 2.5 mm were employed in rate determinations. Colonies of the same size were used because such colonies have undergone approximately the same number of cell divisions. Each colony was resuspended in sterile distilled water and plated on synthetic complete medium, leucineless tryptophanless medium, cycloheximide medium and leucineless tryptophanless cycloheximide medium.

Induction of Rec mutations by ultraviolet light mutagenesis. The LBL1 strain was plated on leucineless tryptophanless medium and exposed to a 65% killing dose of ultraviolet light. Surviving red colonies of 2.5 mm diameter were resuspended and plated on leucineless tryptophanless cycloheximide medium to detect rec mutations affecting spontaneous gene conversion and intergenic recombination. Red cycloheximide resistant segregants of LBL1 detected on leucineless trptophanless cycloheximide medium result from gene conversion of $CYH2^S$ to $cyh2^r$ while white resistant colonies result from intergenic recombination in the CYH2 - TRP5 interval (5).

RESULTS

Chromosomal Recombination and Segregation in Strain LBL1

Strain LBL1 is a n + 1 hyperhaploid disomic for chromosome VII which we employed for the isolation of hyper-rec and hypo-rec mutations affecting mitotic gene conversion and/or intergenic recombination. The genotype of LBL1 is shown in Figure 1. The disomic chromosome is heterozygous at six loci distributed on both sides of the centromere. The strain forms red colonies or chemically defined and complex glucose nutrient media due to the presence of the ade2-1 mutation that confers adenine auxotrophy and accumulation of a cell-limited red pigment (15). The trans arrangement of markers at LEU1 and TRP5 is employed for maintenance of the disomic strain. Haploid segregants

resulting from breakdown of the disome are unable to grow on media lacking
leucine and tryptophan. The cyh2^r mutation, a recessive marker, permits
simultaneous monitoring of gene conversion, intergenic recombination and
restitution of haploidy.

Gene conversion, intergenic recombination and restitution of haploidy (due
to chromosomal loss of nondisjunction) result in rare cycloheximide resistant
segregants that can be detected selectively as summarized in Table 1 and
previously reported (5). The rates of occurence of each class of mitotic event
resulting in cycloheximide resistant segregants were determined by the method
of the median of Lea and Coulson (12) as described in the previous section.
·Twenty-five colonies of the LBL1 strain grown at 24°C were plated on diagnostic
media to determine the number and types of cycloheximide resistant segregants
that had arisen during growth. The results of this analysis are summarized in
Table 2. The data demonstrate that the chromosome VII disome is highly stable
and that gene conversion of CYH2^S to cyh2^r and intergenic recombination in
the interval CYH2 - TRP5 can be detected separately from events resulting in
restitution of haploidy by plating cells on leucineless tryptophanless cyclo-
heximide medium. Two classes of cycloheximide resistant colonies (Classes B
and C of Table 1) arise on this medium. Red colonies are of the Class B type
and are due to gene conversion of CYH2^S to cyh2^r with maintenance of
disomy. White colonies are of the Class C type and result from intergenic
recombination in the interval CYH2 - TRP5. They are white due to homozygosity
for the ade5 mutation which blocks accumulation of the red pigment precursor.

Ultraviolet light induced rec mutations affecting spontaneous mitotic gene
conversion, as measured by the appearance of Class B segregants, and/or
intergenic recombination, as measured by the appearance of Class C segregants,
were obtained as described below.

Isolation of Recombination Mutants

The LBL1 strain was plated on leucineless tryptophanless medium and
mutagenized by exposure to ultraviolet light as described in MATERIALS AND
METHODS. After growth at 24°C, surviving red colonies were resuspended and
plated on leucineless tryptophanless cycloheximide medium to assay the
frequencies of Class B and Class C segregants within each colony. A total of
2,467 survivors of UV mutagenesis were examined. Fifty-four mutants that
exhibit a reproducible hypo-rec or hyper-rec phenotype were obtained. The
phenotypes of the mutants are summarized in Table 3. Five phenotypic groups
were observed. Mutants in Group I exhibit reduced levels of both spontaneous
mitotic gene conversion and intergenic recombination. This mutant class
demonstrates that gene conversion and intergenic recombination are under
coordinate genetic control. Group II mutants retain the capacity for gene
conversion but exhibit a reduced level of intergenic recombination. The
existence of this class indicates there are REC gene products required for
detection of intergenic recombination that are not required for gene
conversion. Mutants of Group III display normal levels of spontaneous gene
conversion and enhanced intergenic recombination. The existence of Group III,
like that of Group II, demonstrates that intergenic recombination is affected
by gene products that are not involved in gene conversion.

Table 1. Chromosomal loss, nondisjunction, gene conversion and intergenic recombination of chromosome VII markers.

Event	Genotypes of cycloheximide resistant segregants		Phenotypic class[a]
		ade5 cyh2^r trp5 LEU1 ade6 cly8	
Chromosomal loss or nondisjunction	Haploid	ade5 cyh2^r trp5 LEU1 ade6 cly8	A
Gene conversion of CYH2^s to cyh2^r	Disome	ADE5 cyh2^r TRP5 leu1 ADE6 CLY8 / ade5 cyh2^r trp5 LEU1 ade6 cly8	B
Recombination in the interval CYH2 - TRP5	Disome	ade5 cyh2^r TRP5 leu1 ADE6 CLY8 / ade5 cyh2^r trp5 LEU1 ade6 cly8	C
Recombination in the interval TRP5 - LEU1	Disome	ade5 cyh2^r trp5 leu1 ADE6 CLY8 / ade5 cyh2^r trp5 LEU1 ade6 cly8	D
Recombination in the interval LEU1 - centromere	Disome	ade5 cyh2^r trp5 LEU1 ADE6 CLY8 / ade5 cyh2^r trp5 LEU1 ade6 cly8	D
Gene conversion and chromosomal loss or nondisjunction	Haploid	ade5 cyh2^r trp5 LEU1 ade6 cly8	A
	Haploid	ADE5 cyh2^r TRP5 leu1 ADE6 CLY8	E
Recombination and chromosomal loss or nondisjunction	Haploid	ade5 cyh2^r trp5 LEU1 ade6 cly8	A
	Haploid	ade5 cyh2^r trp5 LEU1 ADE6 CLY8	D
	Haploid	ade5 cyh2^r TRP5 leu1 ADE6 CLY8	F
	Haploid	ade5 cyh2^r trp5 leu1 ADE6 CLY8	G

a   Class A is diagnostic of chromosomal loss or nondisjunction. Class B is diagnostic of gene conversion of CYH2^s to cyh2^r with maintenance of disomy. Class C is diagnostic of recombination with maintenance of disomy. Class D is diagnostic or recombination. Class E is diagnostic of gene conversion and restitution of haploidy. Classes F and G are diagnostic of recombination and restitution of haploidy.

Table. rates of spontaneous mitotic restitution of haploidy, gene conversion and intergenic recombination on chromosome VII markers.

Event detected	Phenotypic class	Mitotic rates: Cyh medium	Mitotic rates: Leu⁻Trp⁻Cyh medium
Chromosomal loss or nondisjunction	A	1.37×10^{-5}	
Gene conversion of $CYH2^s$ to $cyh2^r$	B	1.45×10^{-6}	2.03×10^{-6}
Recombination in the interval CYH2 - TRP5	C	3.66×10^{-6}	3.30×10^{-6}
Recombination in the interval TRP5 - ADE6	D	4.47×10^{-6}	
Total of all events[a]		$2.44 \pm 0.24 \times 10^{-5}$	$5.33 \pm 0.64 \times 10^{-6}$

[a] A total of 25 colonies of LBL1 grown on synthetic complete medium at 24°C were plated on both cycloheximide synthetic complete medium and leucineless tryptophanless cycloheximide medium. The total rates per cell division of the ensemble of events that result in Cyh^r and $Leu^+Trp^+Cyh^r$ resistant segregants were determined by the median method of Lea and Coulson (12). A total of 1033 colonies recovered on Cyh medium and 314 colonies recovered on Leu⁻Trp⁻Cyh medium were further characterized to determine the rates for each phenotypic class. Classes A,B,C, and D accounted for 98% of the events detected on Cyh medium. Classes B and C accounted for 98% of the events detected on Leu⁻Trp⁻Cyh medium (5).

Table 5. Mitotic recombination mutants isolated following ultraviolet light mutagenesis of LBL1.

Phenotypic Groups	No. Mutants Obtained	Average Spontaneous Mitotic Recombination Frequencies[a]	
		Cyh^r Conv./10^6 cells	Cyh^r Inter. Rec./10^6 cells
I. Conv.$^-$ Recip. Exch.$^-$	13	1.5	0.8
II. Conv.$^+$ Recip. Exch.$^-$	24	22.8	0.8
III. Conv.$^+$ Recip. Exch.$^{+++}$	13	15.3	121.9
IV. Conv.$^-$ Recip. Exch.$^+$	1	0.8	19.7
V. Conv.$^{+++}$ Recip. Exch.$^{+++}$	3	94.7	1006.4
Control LBL1 (25 colonies)		19.4	29.1

[a]The average values for each phenotypic group of mutants represent the results of four independent determinations per mutant. For each determination a 2.5 mm colony grown on synthetic complete medium was resuspended and plated on leucineless tryptophanless cycloheximide medium to determine the frequency of red $Leu^+Trp^+Cyh^r$ convertants and white $Leu^+Trp^-Cyh^r$ intergenic recombinants. Phenotypic symbols are as follows: $-$ = hypo-rec; $+$ = wildtype; and $+++$ = hyper-rec.

Groups IV and V contain the fewest number of mutants. Group IV, containing one mutant, fails to exhibit conversion but does exhibit intergenic recombination. The phenotype of Group IV demonstrates that gene conversion involves processes that are not required for intergenic recombination. Group V mutants exhibit enhanced levels of both gene conversion and intergenic recombination. Like Group I, Group V demonstrates the existence of REC gene products that coordinately control the levels of gene conversion and intergenic recombination in mitotic cells. More detailed information regarding the functions of the REC genes comprising the five phenotypic classes which we have identified can be obtained by examining their meiotic phenotypes, as discussed below.

DISCUSSION

Several molecular models of recombination have been proposed to explain the properties of mitotic and meiotic gene conversion and reciprocal recombination (2,11,14,16). Most share three elements in common: 1) Gene conversion reflects the establishment of heteroduplex DNA, which may persist or undergo mismatch repair; 2) Reciprocal recombination results from the formation, isomerization, and endonucleolytic cleavage of Holliday structures; and 3) Chromosomal recombination is initiated postreplicationally in G2 of mitosis and meiosis. Recent studies, however, have provided genetic evidence that most, if not all, mitotic recombination between homologous chromosomes of diploid cells is initiated prereplicationally (3,7,18) and that postreplicational mitotic sister chromatid exchange also occurs (17). We proposed a molecular model of prereplicational mitotic recombination to explain the properties of mitotic recombination between homologs (3,9). According to the model prereplicational recombination is initiated by heteroduplex DNA formation involving unreplicated homologs. The formation of heteroduplex DNA results in gene conversion of heterozygous markers and can be detected both with and without mismatch repair (6). Gene conversional events, for example, result in Class B red cycloheximide resistant segregants of the LBL1 disome (Table 1). Mitotic reciprocal intergenic recombination, according to our model, requires the formation of a Holliday structure, does not require isomerization, and can only be detected when the Holliday structure is not endonucleolytically cleaved and persists through chromosomal DNA replication. Class C segregants of LBL1 presumably arise in this manner by formation of a Holliday structure in the CYH2 - TRP5 interval.

The critical functions required for detection of prereplicational mitotic gene conversion and intergenic recombination are summarized in Table 4. The collection of hyper-rec and hypo-rec mutants isolated in the LBL1 strain (Table 3) includes all of the phenotypic groups anticipated following loss of functions affecting prereplicational mitotic gene conversion and reciprocal recombination, i.e., Groups I, II, and III of Table 3. Group IV of Table 3, a rare mutational class, may result in preferential mismatch repair of $CYH2^S$:$cyh2^r$ heteroduplexes to the $CYH2^S$:$CYH2^S$ homoduplex state, thereby diminishing the frequency of Class C red cycloheximide resistant convertants. Group V mutants of Table 3, which increase both gene conversion and intergenic recombination are likely to result from increased overall initiation of mitotic recombination. We have previously described a mitosis-specific hyper-rec mutation of this type, reml-1 (8,9).

Future studies of Group I, II and III rec mutants will focus on a comparison of their mitotic and meiotic phenotypes. Group I is expected to include mutants that are hypo-rec with respect to both meiotic gene conversion and reciprocal recombination owing to loss of a function required for initiation of heteroduplex DNA formation in both mitosis and meiosis (Table 5). Group II mutants are expected to be of two types. Those which diminish Holliday structure formation in both meiosis and mitosis are expected to affect mitotic and meiotic recombination in the same manner (Table 5). Those which are defective in mitotic reciprocal recombination due to failure to resolve mitotic Holliday structures by replication are not expected to affect meiotic reciprocal recombination (Table 5). Group III mutants that exhibit a hyper-rec phenotype with respect to reciprocal recombination due to loss of a function required for both mitotic and meiotic endonucleolytic scission of Holliday structures are expected to exhibit a hypo-rec phenotype with respect to meiotic reciprocal recombination. Evidence that the spoll-1 mutation may belong to this class of rec mutations is discussed in this volume by Bruschi and Esposito.

The isomerization of Holliday structures may play no important role in prereplicational recombination (Tables 4 and 5). Consequently rec mutants isolated in LBL1 are not expected to include variants defective in isomerization of Holliday structures. Putative isomerase defective mutants can, however, be detected as variants that fail to exhibit reciprocal sister chromatid exchange and reciprocal meiotic intergenic recombination. Selective systems to isolate such mutants are presently available.

Table 4. Prereplicational versus postreplicational recombination.

Functions involved in recombination	Prereplicational recombination	Postreplicational recombination
1. Formation of heteroduplex DNA	required	required
2. Establishment of Holliday structures	required	required
3. Isomerization of Holliday structures	not required	required
4. Endonucleolytic scission of Holliday structures	not required and antagonistic	required
5. Replicational resolution of Holliday structures	required	impossible
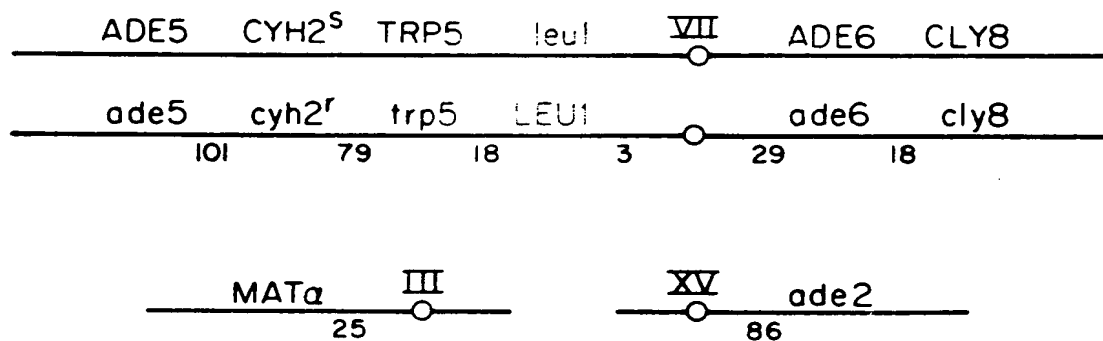
Figure 1. Genotype of the chromosome VII disomic strain LBL1. The map distances shown are the standard map of the value.

Table 5. Predicted phenotypes of rec mutations affecting prereplicational and/or postreplicational recombination[a].

Absent function	Prereplicational recombination		Postreplicational recombination	
	Conversion	Recip. Exch.	Conversion	Recip. Exch.
1. Formation of heteroduplex DNA	-	-	-	-
2. Establishment of Holliday structures	+	-	+	-
3. Isomerization of Holliday structures	+	+	+	-
4. Endonucleolytic scission of Holliday structures	+	+++	+	-
5. Replicational resolution of Holliday structures	+	-	+	+

[a]Phenotypic symbols are as follows: - = hypo-rec; + = wildtype; and +++ = hyper-rec

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19. RECOMBINATION PROCESSES IN A SPORULATION-DEFECTIVE MUTANT OF S. cerevisiae: ROLE OF HOLLIDAY STRUCTURE RESOLUTION*

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A mutant of the yeast Saccharomyces cerevisiae, defective in the production of ascospores, is used for comparative analysis of the formation and resolution of Holliday structures in meiotic and mitotic recombination. Diploid hybrids homozygous for the mutation spoll-1 exhibit not only a sporulation-deficient phenotype, but also a very low level of ascosporal viability, due to nondisjunction and chromosomal loss. Ascosporal survival is ca. 0.02% while in SPO11/SPO11 diploids ascosporal survival typically exceeds 95%. The genetic characterization of spoll-1/spoll-1 hybrids has provided evidence supporting the hypothesis that failure to cut Holliday structures, during both mitosis and meiosis results in a hyper-rec phenotype in mitosis and hypo-rec phenotype in meiosis. Diploid spoll-1/spoll-1 cells are hyper-rec with respect to spontaneous mitotic intergenic recombination and hypo-rec with respect to spontaneous meiotic intergenic recombination. The spontaneous level of mitotic intergenic recombination at 24°C, a semi-permissive temperature for the sporulation process in spoll-1/spoll-1 hybrids, is ca. 5 fold higher in spoll-1/spoll-1 than in wild-type SPO11/SPO11 hybrids, while no significant difference exists at the restrictive temperature of 34°C. No activity on spontaneous intragenic heteroallelic recombination (gene-conversion), which does not depend upon resolution of Holliday structures, is detected at both temperatures. Meiotic intergenic recombination in the mutant, in contrast, is ca. 2 fold lower than that of wild type hybrids.

The phenotype of spoll-1 homozygous diploids precisely fulfills the expected phenotype predicted according to the model of prereplicational mitotic recombination versus postreplicational meiotic recombination for cells defective in the endonucleolytic scission of Holliday structures in both mitosis and meiosis. Preliminary studies of mitotic and meiotic recombination of the 2-micron DNA plasmid in spoll-1/spoll-1 and SPO11/SPO11 cells have been initiated to characterize the recombinational defect of spoll-1 cells at the DNA molecular level.

INTRODUCTION

Different mechanisms of genetic recombination in meiosis and mitosis and the existence of common REC gene functions can be genetically studied in yeast by the isolation of mutants affected in one or both processes with respect to the formation and resolution of Holliday structures (7). In meiosis, in which genetic recombination appears to occur after DNA synthesis and chromosomal duplication, in the G2 phase, the cleavage of Holliday structures formed in the G2 phase is the hypothesized basis for the generation of meiotic intergenic recombinants (11,15). Failure to resolve Holliday structures that engage nonsister chromatids would be expected to reduce the level of detectable meiotic intergenic recombination, to cause extensive meiotic nondisjunction and to result in drastic ascospore lethality owing to the consequent aneuploidy.

The effect of failure to resolve Holliday structures in mitosis is expected to differ from that observed in meiosis. We have recently presented genetic data indicating that spontaneous mitotic recombination between homologous chromosomes occurs at the two-strand stage, i.e., in the G1 phase, before chromosomal duplication, in both wild type hybrids and hybrids homozygous for the mitotic hyper-rec mutation reml-1 (6,10). A specific prereplicational model of mitotic recombination at the two-strand stage was advanced by Esposito (6), who proposed that intergenic recombination is initiated pre-replicationally by formation of Holliday structures. Some of these structures, that are not resolved by endonucleolytic scission, are instead resolved by chromosomal DNA replication (Figure 1). According to this model, failure to cut Holliday structures by enzymatic activity before DNA replication would allow more of them to be resolved by DNA synthesis and would increase the level of mitotic recombination. These resolution events change the linkage relationships of heterozygous markers bracketing the site of the Holliday structure and are detected by standard methods which rely on the appearance of homozygous sectors. Holliday structures resolved by endonucleolytic cleavage, in contrast, do not produce sectors or mitotic intergenic recombinants and thus escape detection.

A critical analysis of the involvement of resolution of Holliday structures in meiotic and mitotic recombination processes can thus be carried out with mutants that show levels of lethality of the meiotic products due to non-disjunction and consequent chromosomal loss leading to aneuploidy. Such mutants may include variants defective in the resolution of Holliday structures formed during both mitosis and meiosis. They would be expected to exhibit a hypo-rec phenotype with respect to spontaneous intergenic recombination and a hyper-rec phenotype with respect to spontaneous mitotic intergenic recombination. Furthermore, the level of gene-conversion measured as intragenic heteroallelic recombination should be normal, since conversion depends upon formation of heteroduplex DNA rather than the mode of Holliday structure resolution. The extent of mitotic hyper-rec activity of such mutants would depend upon the efficiency with which Holliday structures are normally cleaved in the wild type.
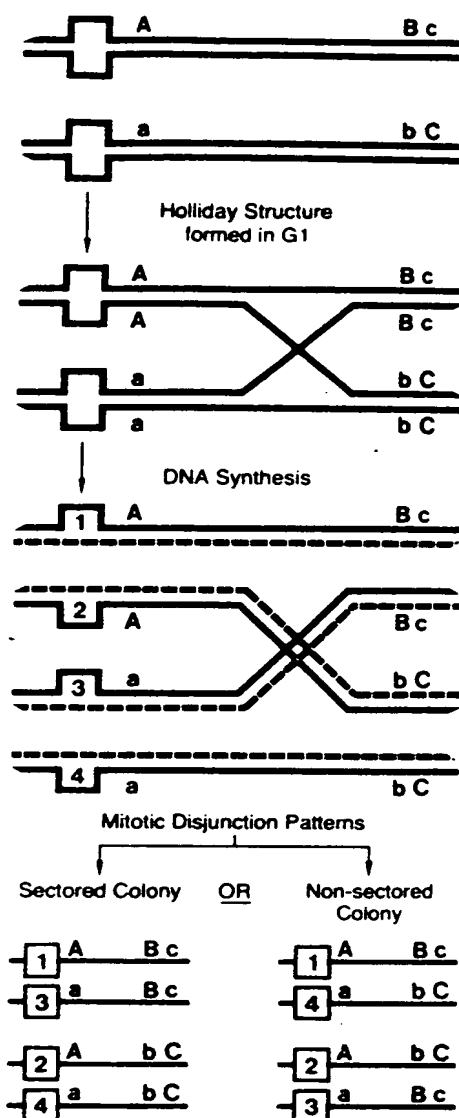
Figure 1. The prereplicational model of mitotic recombination proposed by Esposito (6). Prior to chromosomal duplication, a Holliday structure (11) is formed by exchange of single strands of DNA of the same chemical polarity. When the Holliday structure is not cleaved prior to duplication of the chromosomes the resulting chromatids consist of a pair with markers in the non-recombinant configuation, i.e. 1 and 4 and a pair of recombinant chromatids, i.e. 2 and 3. When chromatids 1 and 3 separate from 2 and 4 a sectored colony results, exhibiting segregation of the recessive markers b and c. The arrows indicate the 3' termini of the deoxyribonucleotide chains. The broken lines indicate DNA synthesized during the S phase of mitosis.

The data described below demonstrate that diploids homozygous for the mutation spoll-1 exhibit the genetic characteristics expected for cells defective in both mitotic and meiotic endonucleolytic scission of Holliday structures.

MATERIALS AND METHODS

Strains. Mitotic and meiotic recombination was studied in the MATa/MATα heterothallic spoll-1/spoll-1 hybrid, CBX1, and the MATa/MATα heterothallic SPO11/SPO11 congenic hybrid, CBX2, háving the genotypes shown in Table 6.

Media and genetic techniques. Genetic procedures and the compositions of the glucose nutrient (YPD), synthetic growth media, and sporulation media have been reported (8,14).

Plating of parallel vegetative cultures. The diploids used in recombination experiments were plated on YPD medium and incubated at 24°C or 34°C to obtain single colonies. 100 ml liquid YPD cultures supplemented with 60 mg/liter of adenine were inoculated with one colony of approximately 3 mm in diameter (ca. 1.5 - 3.0 x 10^7 cells). The cultures were grown at 24°C or 34°C, respectively, harvested during the exponential phase of growth, concentrated by centrifugation, washed once in 10 ml of sterile distilled water and plated on the appropriate synthetic media. The plates were incubated for 7 days prior to counting.

Caclulation of recombination rates. Mitotic recombination rates were calculated by the methods of Drake (1) and Lea and Coulson (13). The pertinent recombination rates for each culture were obtained from the equation $r=0.4343$ $f/(\log N - \log N_0)$, (where r=rate, f=frequency of recombinants, N=final total cell number, and N_0=initial cell number). The median rate was then determined.

RESULTS

Isolation and Properties of spoll-1

The spoll-1 mutation was isolated following ultraviolet mutagenesis of ascospores derived from a homothallic diploid strain of Saccharomyces cerevisiae and subsequently introduced into heterothallic stocks (2,3). Diploids homozygous for the spoll-1 mutation exhibit reduced sporulation in comparison to wild type diploids at both 24°C and 34°C. At 24°C spoll-1/spoll-1 diploids exhibit ca. 25% asci in comparison to wild type diploids which yield ca. 70% asci at this temperature. At 34°C spoll-1/spoll-1 diploids yield ca. 10% asci in comparison to congenic wild type diploids that exhibit ca. 60% sporulation.

We have characterized the effect of the spoll-1 mutation on mitotic and meiotic recombination at both 24°C and 34°C in order to determine the temperature dependence of the spoll-1 defect. To perform the analysis it was necessary to determine the effect of the spoll-1 mutation on ascosporal

viability at 24°C. In a preliminary experiment we dissected 81 four-spored asci and 1 three-spored ascus obtained following sporulation of the spoll-1/spoll-1 hybrid at 24°C. None of the ascospores gave rise to viable colonies at 24°C. This result indicated the spoll-1 mutation results in drastically reduced ascospore viability.

In order to further assess the effect of the spoll-1 mutation on survival of cells exposed to sporulation medium, a sporulated suspension of the spoll-1/spoll-1 hybrid was diluted and plated on solid YPD medium. A total of 1026 plated cells plus asci were observed microscopically over a 20 hr period during incubation at 24°C on YPD plates. The results of this microscopic analysis are summarized in Table 1 below.

Table 1. Viability of spoll-1/spoll-1 after sporulation at 24°C.

Micro-Colonies	Dead Cells	Dead Asci	Total Observed
41	728	257	1026
(4.0%)	(71.0%)	(25.0%)	

Only 41/1026 surviving microcolonies were observed. This observation demonstrates that the spoll-1 mutation results in reduced viability of unsporulated as well as sporulated cells. To obtain a more precise estimate of ascosporal viability of spoll-1/spoll-1 segregants, a total of 855 microcolonies were characterized by light microscopic observation in order to distinguish between apparently diploid cell colonies and haploid cell colonies. Haploid cells can be recognized by their smaller size and haploid growth habit. A total of 4/855 presumptive haploid colonies were observed. Subsequent genetic analysis of putative haploids has supported the conclusion that they are indeed haploid or hyperhaploid. These observations and the knowledge that the sporulated suspension consisted of 25% asci allow us to calculate that ascospore survival of the spoll-1/spoll-1 haploid is ca. 0.02%.

Effect of the spoll-1 Mutation on Meiotic Recombination

We employed the CBX1 spoll-1/spoll-1 hybrid to determine the effect of the spoll-1 mutation on meiotic intergenic recombination for markers on chromosome VII following sporulation at 24°C. Owing to the fact that the spoll-1 mutation drastically reduces ascospore viability, it was not possible to observe its effect upon meiotic recombination by standard tetrad analysis. We performed a modified random spore analysis taking advantage of the fact that the diploids are canl/CAN1 heterozygotes sensitive to canavanine. Meiotic canl segregants resistant to canavanine can be randomly selected out of sporulated populations by replica plating on arginineless plates containing 60 ug/ml of canavanine. ... independent colonies of the spoll-1/spoll-1 hybrid were sporulated and replicated on canavanine medium. Single canavanine-resistant colonies were

MEIOTIC   MAPS

40 Centimorgans

$$\frac{spo11-1}{spo11-1}$$

$$\frac{SPO11}{SPO11}$$

MITOTIC   MAPS

I Mit. event / 10^4 Mitoses

$$\frac{spo11-1}{spo11-1}$$
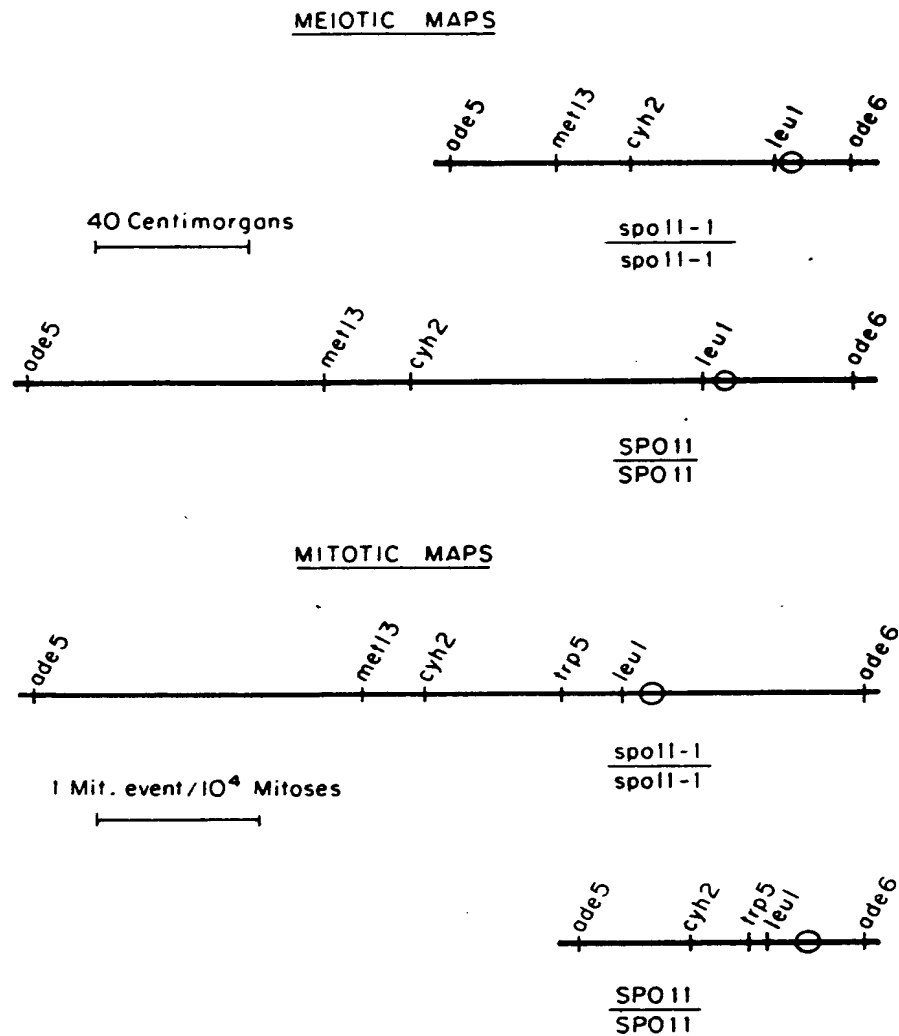
$$\frac{SPO11}{SPO11}$$

Figure 2.   Meiotic and mitotic maps of chromosome VII intervals of the wild
type SPO11/SPO11 and the spo11-1/spo11-1 mutant hybrids.   Both maps of the
wild type were determined in previous studies from our laboratory (8,9,10,14).

then picked up randomly, grown on YPD master plates, replicated to the complete and drop-out media and mated to 4 different ade1 tester strains. Two of these were a and α mating type tester strains and the two others were chromosome VII testers. Mating between the tester strains and spoll-1 meiotic segregants resulted in adenine prototrophic colonies that could be scored following replica plating to adenine-deficient medium. We analyzed 1160 colonies that mated with one of the two mating type testers. The sample included both monosomic chromosome VII segregants and disomics for chromosome VII. The analysis provided an estimate of the percent meiotic recombination among surviving ascospores of the spoll-1/spoll-1 hybrid for the intervals between the chromosome VII markers ade5-met13-cyh2-leul-ade6 and the chromosome V markers can1-hom3. The results are shown in Table 2.

Table 2.   Single strand analysis of meiotic segregants of the spoll-1/spoll-1 hybrid (CBX1)[a]

Interval	+		+		+	−		−	+		−	−		Total	Corrected % Rec.
ade5-met13	R_1	223	P_2	139	P_1	367	R_2	94	823	28
met13-cyh2	R_1	117	P_2	429	P_1	162	R_2	61	769	19
cyh2-leul	R_1	80	P_2	199	P_1	370	R_2	120	769	38
leul-ade6	P_1	406	R_1	86	R_2	68	P_2	261	821	20
can1-hom3	P_1	1083	R_1	77					1160	9

[a] P_1 and P_2 are the parental categories; R_1 and R_2 are the recombinant ones. The percentages of recombination have been corrected for the presence of disomics for chromosome VII that increase the real number of chromosomes involved in the analysis. P_2 and R_2 for the interval between can1 and hom3 on chromosome V are not given because canavanine sensitive strands are not rescued.

On the basis of the values of meiotic recombination reported in Table 2, we have constructed the meiotic map of the spoll-1/spoll-1 hybrid which is compared with that of the SPO11/SPO11 wild type in Figure 2. As one can see there is an overall reduction of ca. 50% of the map length between the ade5 and ade6 markers: the total length of the wild type map is 216 cM while in the mutant it is only 105 cM. The reduction of recombination is not homogeneous in the various intervals measured on chromosome VII. Recombination in the leul-ade6 and cyh2-leul intervals is 50% of the wild type level and 64% of the

wild type level between ade5 and met13. The greatest reduction was observed
in the interval between the can1 and hom3 markers on chromosome V in which the
spoll-1 value is of the order of 14 fold lower than that of the wild type.
The interval met13-cyh2 on chromosome VII exhibited no apparent reduction in
spoll-1/spoll-1.

The gene to centromere distance for leu1 was also estimated but by a
different approach, since it cannot be obtained from random spore analysis.
We isolated a total of 104 microcolonies following sporulation and growth of
the spoll-1/spoll-1 hybrid on YPD medium. In this sample, 98 microcolonies
consisted of diploid cells i.e., they were non-maters, were able to sporulate
poorly, and exhibited heterozygosity for one or more markers originally present
in heterozygous or heteroallelic condition. Among these 98, two exhibited
reciprocal recombination in the leu1 to centromere map interval, providing an
estimate of the meiotic recombination frequency for this interval. Previous
studies have demonstrated that map values obtained among diploids following
extensive exposure to sporulation conditions approximate true meiotic map
distances (4,5,16).

Effect of the spoll-1 Mutation on Mitotic Recombination

The spontaneous rates of mitotic intergenic recombination for markers on
chromosome VII were measured by plating aliquots from five parallel cultures
of the spoll-1/spoll-1 CBX1 hybrid and the SPO11/SPO11 CBX2 congenic wild type
on synthetic complete medium and selective media.

The rate of heteroallelic (intragenic) recombination at trp5, located on
chromosome VII, at 24°C was determined from the total number of prototrophs in
each of the five parallel cultures as described in MATERIALS AND METHODS. The
rate of heteroallelic recombination leading to prototrophy is the same in the
spoll-1/spoll-1 hybrid and the wild type. The spoll-1 mutation does not confer
a hyper-rec phenotype with respect to heteroallelic intragenic recombination
events leading to prototrophy (Table 3). The spoll-1 mutation does, however,
exhibit an enhancement of the rate with which heteroallelic recombination at
trp5 is associated with intergenic recombination in the interval trp5-cyh2
leading to the production of red-white and white Trp$^+$ colonies. The spoll-1
mutation also confers hyper-rec activity in the case of intergenic recombina-
tion events unselected with respect to association with gene conversion, as
discussed below.

The rates of mitotic events resulting in the production of canavanine
resistant segregants at 24°C and of cycloheximide resistant segregants at 24°C
and 34°C are reported in Tables 4 and 5, respectively. No substantial
differences between spoll-1/spoll-1 and SPO11/SPO11 were observed. Since
canavanine resistant and cycloheximide resistant segregants arise by both
conversion and intergenic recombination in unknown proportions we proceeded to
measure intergenic recombination on chromosome VII.

Intergenic recombination on chromosome VII was monitored by the production
of white and red-white sectored colonies on synthetic complete medium at the
two temperature of 24°C and 34°C. Since the hybrids are homozygous for the
ade2-1 mutation, mitotic events that result in homozygosity for either ade5 or

Table 3. Spontaneous tryptophan prototrophic segregants recovered from cultures following growth of the spoll-1/spoll-1 and SPO11/SPO11 hybrids at 24°C.

Diploid	Culture Number	Prototrophic segregants, No/10^6				Cells/culture x 10^{-9}
		Red	White	Sectored	Total	
spoll-1						
spoll-1	1	16.9	65.2	0.0	82.2	1.56
	2	18.9	3.4	1.0	23.2	1.03
	3	6.7	2.2	0.0	8.9	1.19
	4	18.9	3.3	0.0	22.1	1.22
	5	22.5	111.0	0.0	133.5	1.18
	6	28.1	2.2	0.1	30.4	3.19
	7	16.3	1.1	0.2	17.5	5.95
	8	18.6	3.0	0.1	21.7	4.98
	9	21.2	4.9	0.0	26.0	3.39
	10	14.4	2.1	0.5	17.0	4.28
	Rate	1.00 x 10^{-6}	1.70 x 10^{-7}		1.27 x 10^{-6}	
SPO11						
SPO11	1	20.3	1.4	0.0	21.7	3.42
	2	16.2	4.2	0.0	25.3	2.51
	3	19.2	2.5	0.0	21.7	3.69
	4	111.9	1.5	0.2	113.6	7.83
	5	233.1	2.2	0.4	235.7	3.02
	Rate	0.93 x 10^{-6}	1.11 x 10^{-7}		0.99 x 10^{-6}	

Table 4.   Spontaneous canavanine resistant segregants recovered from cultures following growth of spo11-1/spo11-1 and SPO11/SPO11 hybrids at 24°C.

Diploid	Culture Number	Resistant segregants, No/10^5				Cells/culture x 10^{-9}
		Red	White	Sectored	Total	
spo11-1/spo11-1	1	35.8	0.3	1.5	37.6	1.56
	2	31.0	2.3	0.0	33.3	1.03
	3	39.3	0.0	0.0	39.3	1.19
	4	142.0	2.3	0.3	144.6	1.22
	5	54.2	8.8	0.0	63.1	1.18
	6	23.2	0.0	0.0	23.2	3.19
	7	42.2	0.1	0.0	42.3	5.95
	8	25.6	0.0	0.0	25.6	4.98
	9	26.0	0.3	0.1	26.5	3.39
	10	33.7	0.0	0.3	34.0	4.28
	Rate	1.90×10^{-5}	1.50×10^{-7}		1.97×10^{-5}	
SPO11/SPO11	1	17.0	0.6	0.0	17.6	3.42
	2	38.2	14.9	0.0	53.1	2.51
	3	38.0	0.4	0.0	38.4	3.69
	4	29.4	0.2	0.0	29.6	7.83
	5	151.8	349.7	9.5	511.0	3.02
	Rate	1.76×10^{-5}	3.00×10^{-7}		2.99×10^{-5}	

Table 5. Spontaneous cycloheximide resistant segregants recovered from
colonies of the spoll-1/spoll-1 and SPO11/SPO11 hybrids grown
at 24°C and 34°C.

Diploid	Colony Number	Colonies grown at 24°C		Colonies grown at 34°C	
		Resistant segregants No. 10^5	Cells/colony $\times10^{-7}$	Resistant segregants No. 10^5	Cells/colony $\times10^{-7}$
spoll-1 spoll-1	1	12.3	2.6	24.1	3.9
	2	18.6	2.2	18.0	1.8
	3	13.5	2.5	18.2	2.4
	4	22.0	2.2	18.0	2.1
	5	10.9	2.6	47.5	1.7
	Rate	$\sim 0.8 \times 10^{-5}$		$\sim 1.1 \times 10^{-5}$	
SPO11 SPO11	1	6.2	1.2	7.0	1.8
	2	7.0	1.4	19.4	0.9
	3	29.6	1.6	19.8	1.9
	4	23.9	0.8	10.1	2.3
	5	10.7	1.6	9.5	2.8
	Rate	0.7×10^{-5}		0.6×10^{-5}	

ade6 result in the production of white and red-white sectored colonies.
Red-white sectored colonies represent events that occurred in the cell
divisions following plating. Their frequency thus provides a direct estimate
of the rate of mitotic events resulting in ade5/ade5 and ade6/ade6 segregants
(Table 6). Comparing the rates of the mutant and the wild type at 24°C we
found a five fold higher rate for the mutant that is statistically significant
at the 99% confidence level. At 34°C, however, there is only a small

Table 6. Mitotic recombination rates of spo11-1/spo11-1 and SPO11/SPO11 hybrids at 24°C and 34°C

Diploids	Temperature	Red-white sectored colonies on synthetic complete	Cyh^r	Trp$^+$	Can^r
spo11-1/spo11-1 (CBX1)	24°C	$5.2 \pm 1.2 \times 10^{-4}$ (19/36821)	0.8×10^{-5}	1.3×10^{-6}	2.0×10^{-5}
	34°C	$5.3 \pm 1.4 \times 10^{-4}$ (14/26668)	1.1×10^{-5}		
SPO11/SPO11 (CBX2)	24°C	$1.4 \pm 0.6 \times 10^{-4}$ (05/34808)	0.7×10^{-5}	0.9×10^{-6}	2.9×10^{-5}
	34°C	$4.3 \pm 1.3 \times 10^{-4}$ (11/25288)	0.6×10^{-5}		

					VII		V		XV	
ADE5	met13-c	cyh2^r	trp5-c	leu1-c	─o─ ade6	hom3 ─o─	CAN1^s	─o─	ade2
ade5	MET13	CYH2^s	trp5-d	LEU1	ADE6	HOM3	can1^r		ade2

difference between the two rates of recombination that is in the range of the standard deviations and is not significant. It thus seems that the wild type gene product is temperature sensitive and that the spo11-1 mutation reduces the range in which the enzymatic activity is optimal. At 34°C, the SPO11/SPO11 hybrid is a phenocopy of the spo11-1/spo11-1 mutant diploid.

The genotypes of 41 red-white sectored spo11-1/spo11-1 colonies were determined to locate the exchanges resulting in homozygosity for the distal markers ade5 and ade6. Five subclones from the red and white portions of the sectored colonies were sporulated at 24°C and backcrossed to haploid chromosome VII testers. Their genotypes were inferred from the phenotypes of the back-cross hybrids. The results of this analysis are summarized in Figure 2, which illustrates the mitotic hyper-rec activity of spo11-1/spo11-1 with respect to intergenic events.

In order to demonstrate the involvement of the resolution of Holliday structures by DNA synthesis at the molecular level during mitotic recombination, we have begun a structural study of the yeast 2-micron DNA plasmid in the spo11-1/spo11-1 hybrid in which we expect the formation of dimers and multimers as a product of DNA replication through uncleaved Holliday structures. Preliminary results obtained by electron-microscopy of covalently closed DNA molecules in spo11-1/spo11-1 diploids did show the frequent presence of dimers and tetramers. Size determinations by . . . electrophoresis are currently in progress.

DISCUSSION

The data presented above provide strong evidence in favor of the conclusion that the product of the SPO11 gene is directly involved in endonucleolytic cleavage of Holliday structures in both mitosis and meiosis. This fact identifies a common step in the mitotic and meiotic pathways of recombination. The mitotic hyper-rec and meiotic hypo-rec phenotype with respect to intergenic recombination of the spo11-1/spo11-1 hybrid lends support to the view that recombination between homologs of mitotic cells occurs prereplicationally while meiotic recombination between homologs occurs postreplicationally. Given these results, genetically marked haploid and diploid spo11-1 strains can now be employed to test a corollary of the G1 model of mitotic recombination, viz, that sister chromatid exchange, like meiotic homologous chromosomal recombination, is dependent upon endonucleolytic cleavage of Holliday structures. Several yeast systems amenable to this type of analysis have already been developed in other laboratories (cf. 7 for review).

The spo11-1 mutation also provides a basic genetic approach to determine whether the overall sequence of molecular events postulated to occur by molecular models of genetic recombination is correct (6). The data discussed above suggest that the SPO11 gene encodes a late function in the recombination sequence. Previous studies have uncovered both hyper-rec and hypo-rec mutations that may affect early stages of recombination, e.g., initiation of single-strand transfer (7). The phenotypes of doubly mutant strains incorporating spo11-1 and those previously isolated rec mutations may challenge models of genetic recombination. Reciprocal temperature shift studies (12) employing ts-rec mutants may also provide information with respect to the order of function in recombination of REC gene products.

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20. THE MOLECULAR GENETICS OF NON-TANDEM DUPLICATIONS AT ADE8 IN YEAST*

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The ADE8 locus of chromosome IV has been cloned. Various subclones, principally those with fragment lengths of 2.5 and 4.0 kb, confer ADE8 function on recipient strains carrying the unique allele ade8-18. A visual screen for detecting integrations of the autonomously replicating vector is described along with diagnostic genetic tests that identify the genomic integration sites. Most integrants generate non-tandem duplications at the ade8-18 site, though some also occur at trp1. None were found at ura3. The frequency of integration via homologous recombination into a genomic site is proportional to the physical length of the corresponding DNA segment, carried in the YRp17 vector. Similarly, overall plasmid excision rates are proportional to the total length of the integrated segment and the distribution of events for a plasmid with a given DNA insert is determined by the position of the mutant site within the genetic fine structure map. Meiotic gene conversion, intrachromosomal conversion, and postmeiotic segregation were studied in several hybrids containing two, three or four ADE8 sequences within conventional chromosomes or non-tandem duplications that are either isosequential or heterosequential.

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Gene conversion is typically defined as a non-reciprocal transfer of genetic information from one parental DNA segment to the corresponding DNA segment of the homologous chromosome (10-17,21,25). In yeast, it is generally agreed that conversion events can occur both in meiosis and mitosis. The meiotic process involves specific synaptic pairings at the zygotene or pachytene stage of prophase I, along with the formation of heteroduplex DNA. These heteroduplexes may isomerize, as suggested by the Aviemore model (24,28) to yield associated exchanges that recombine the outside markers. Alternatively, isomerization might not occur and the somewhat more probable parental marker array is maintained. Thus, because gene conversion and genetic recombination are mechanistically linked, conversion has been viewed as a signal of the basic recombination event (15,16,17).

More recently, the definition and significance of gene conversion has been enlarged and extended—particularly by perspectives and insights stemming from the experimental potentialities inherent in recombinant DNA technology coupled to yeast transformation (2). Several major studies impinge on current molecular recombination schemes and these are based on a combined genetical-recombinant DNA approach. The importance of these works is summarized here.

Introduced into sites on non-homologous chromosomes, by means of recombinant DNA procedures, various his3 alleles were shown to undergo heteroallelic recombination and restoration of prototrophy (31). Clearly, homologous sequences could experience informational transfer or gene conversion regardless of their position in the genome. Of course, the frequency of such heterologous events is several orders of magnitude lower than controls involving heteroalleles occupying corresponding sites on homologous chromosomes. Similar findings were reported by others (1).

Related to this extension of gene conversion's definition are the reports concerning sister chromatid exchange (23,34). Such studies were facilitated by the coupling of critical genetic analyses with recombinant DNA procedures that permitted differential marking of highly iterated sequences. To account for the events observed, as well as the more recently described instance of gene amplification at the cup1 locus in yeast, it is assumed that misalignment between homologous iterated segments is followed by heteroduplex DNA formation, isomerization and an appropriate scission (4). Taken collectively, these events lead to a reciprocal exchange that generates quantitatively dissimilar products i.e., unequal crossing over. This event sequence may occur in mitotic cells and involve sister chromatids. Alternatively, it could occur in meiotic cells where homologous chromosomes might be engaged interactively during synapsis. With regard to DNA replication, conversion is viewed as prereplicative in mitosis (7,8,9,18) and as postreplicative in meiosis (2-4).

A final approach entails the analysis of non-tandem duplications. These originate as a consequence of integrational events, i.e. homologous recombination events which lead to the incorporation of a plasmid bearing a cloned DNA sequence into a site within the corresponding genomic region. Several studies (19,20,22,23) may be taken as representative. Such genomic reorganizations allow for the possibility of intrachromosomal gene conversion with and without associated crossing over when the interactive sequences are separated by a physical distance equal to the number of base pairs contained in the cloning vehicle.

The present study aims to clone, analyze and sequence the structural and regulatory sequences of the ARG4 and ADE8 loci. Of special interest are the alleles arg4-16, ade8-14, ade8-18 and others for which we have accumulated an extensive, highly detailed background of biochemical and recombinational data (5,6,8,15,16,17). Our program seeks to address, at the DNA sequence level, the generalizations based on genetic analysis that relate to parity, fidelity, polarity, frequencies of associated outside marker exchange, the position of associated exchanges, the conversional behavior of insertions, deletions and perhaps most importantly the marker effects of specific mutants (5,17). Marker effects may refer to DNA structural-organizational features relating to

1. Fogel and Welch, in press, PNAS September 1982.

heteroduplex DNA correction mechanisms that sense, detect and repair mismatched base pairs as in single base substitution or frameshift mutants, or extensive non-homologies as are found at the MAT locus, or non-pairings as in deletions or insertions several kilobases in extent (16).

The production of non-tandem duplications and some of their salient features have been reported (19,20,22,23). The present approach seeks to exploit the resolving power of recombinant DNA-transformation systems with a view towards advancing our understanding of recombination from the self-contained black box level of genetic fine structure analysis to the physical-molecular level. Current state of the art techniques make it practical to isolate and analyze specific genes or gene fragments (2). Moreover, isolated genes, carried in plasmid vectors with known but varied replicative and integrative behavior, may be subjected to in vitro or in vivo mutagenesis and then returned to the host cell by DNA transformation for studying the effects of such alterations on gene organization, expression and regulation.

Cloning the ADE8 locus

A recipient conventional diploid strain of the following genotype was synthesized after several rounds of mating, sporulation, ascus dissection, scoring ascosporal colonies and selection. Except for MAT, each of the following markers was homozygous ade2-1 trpl, ura3 ade8-18 arg4-16, cupl. Comparable strains carrying the wild type allele at ade2 were also prepared. When these studies were initiated by the senior author, in the laboratory of R.W. Davis at Stanford University, it was presumed that transformation studies in diploids immediately amenable to meiotic characterization would confer several advantages over transformation in haploids. Accordingly, these strains were monitored and selected for abundant sporulation with a high proportion of 4-spored tetrads, high ascosporal survival equal to or better than 95%, and ease of spheroplasting and transformability. From a vantage point of hindsight, it is apparent that congenic haploids appropriately marked to facilitate subsequent analytical studies would also be useful.

A random library of DNA fragments was prepared from the DNA isolated from a standard yeast strain—S288C. Conventional restriction enzyme digestion and sticky end ligation were involved. Yeast DNA was partially digested with the 6 base pair recognition enzyme BamHI and the fragments were ligated into the unique BamHI site of the YRp17 vector. This plasmid was constructed and kindly made available in purified form by W.M. McDonnell and R.W. Davis. The plasmid YRp17 shown in Fig. 1 is derived from YRp12. It contains a unique BamHI site located within the tetracycline resistance gene. This site is also present in YRp7, a TRP1/pBR322 vector (33). YRp17 also harbors the yeast URA3 locus, ARS1—an autonomous replication sequence, and an adjacent, unique EcoRI site (32). Pools containing about 25,000 independent plasmids, i.e. each plasmid bearing a different inserted DNA fragment, were obtained by transforming E. coli to ampicillin resistance and tetracycline sensitivity. The pool's informational content was assessed by transforming the above described recipient yeast strain and selecting for complementation with respect to the TRP1 and URA3 functions. Among the latter, colonies displaying complementation relative to ade8-18 could be readily identified in the recipient ADE2 strain, since their replica-transfer prints would grow confluently on synthetic complete media deficient for adenine. Single yeast colonies containing presumptive ADE8 strain DNA were purified, grown, lysed and used to infect E. coli
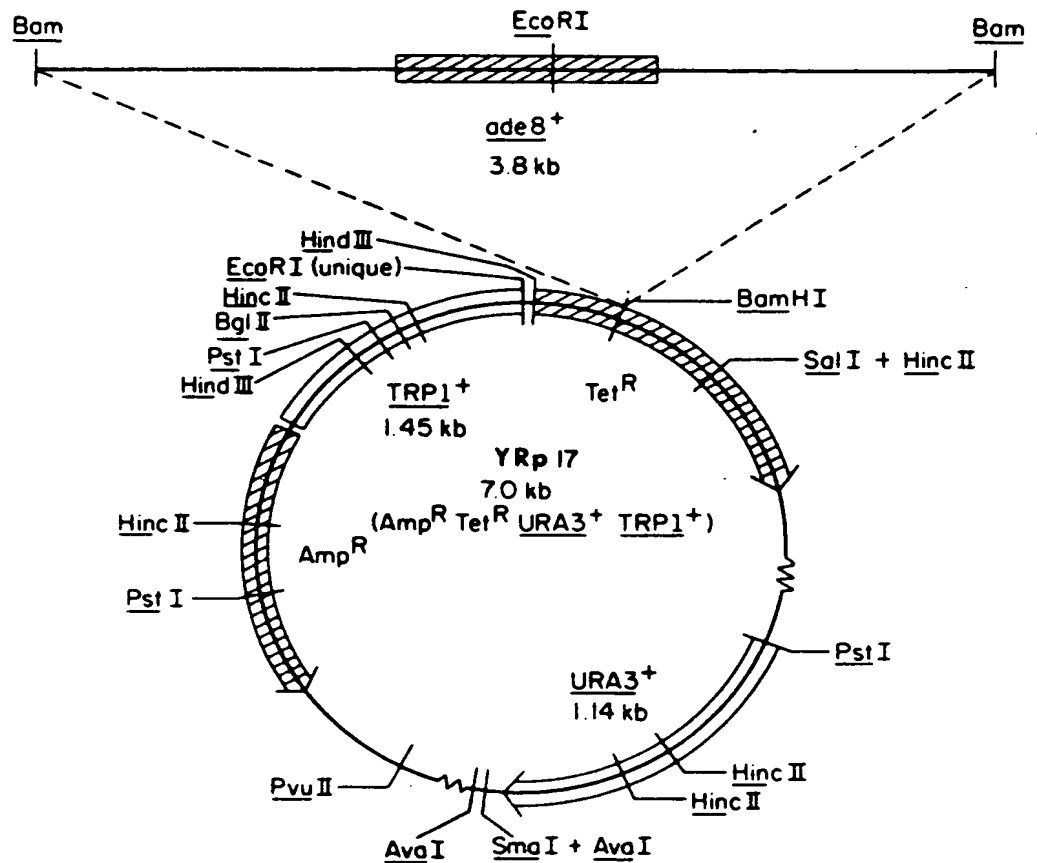
Fig. 1. The YRp17 cloning vehicle (constructed by W.M.McDonnell and R.W. Davis, pers. comm.) is a 7.0 kb plasmid carrying pBR322 sequences and the wild type sequences for the yeast genes TRP1 and URA3 in addition to an autonomous replication sequence, ars. A 4.0 kb BamHI insert carrying ADE8 function is shown.

cultures for the isolation of purified plasmid preparations. A YRp17 plasmid containing a 4kb wild type DNA fragment inserted into the unique BamHI site comprises the initial hybrid DNA species on which the present study is based.

The 4kb fragment was subjected to restriction enzyme digestion, electrophoretic analysis, and subcloning. The restriction map is given as Fig. 2. Several different subclones were isolated and two will be discussed here. The first is about 2.5 kb in length. It was generated by cleaving the 4kb BamHI fragment with the enzyme EcoRl—a procedure that yields two fragments; i.e., 2500bp and 1500bp. When these were subcloned and tested, only the larger fragment provided ADE8 function. Like the parental 4.0 kb segment, it integrates preferentially into the chromosome IV region marked by ade8-18. A smaller, secondary subclone of 1750 bp (from SalI to BamHI shown in Fig. 2) fails to provide ADE8 function in URA3, TRP1 transformants. But, the subclones of the 2500bp fragment recombine intragenically within the ade8-18 sequence, at an extremely low frequency, to yield functional ADE8 prototrophs. Thus, the coding region and probably the regulatory sequence of ADE8 are available to us within a combined physical length corresponding to about 2500 bp. The ade8-18 site falls within the 1750 bp fragment. In contrast, the mutant, ade8-10, is located within a cluster adjacent to a terminus of the ADE8 genetic fine structure map (5). Recombinational integration resulting in prototrophy occurs between this ochre nonsense mutant and the smaller 750 bp segment. Accordingly, the 750 bp fragment includes ade8-10 but not ade8-18, since it recombines only with the former heteroallele, while the larger 1750 fragment must subtend and include the non- revertible, high PMS ade8-18 site situated in the approximate center of the fine structure map; see Fig. 3. A DNA sequence analysis of the 1750 bp fragment containing the mutant ade8-18 compared to wild type will almost certainly provide a molecular basis that accounts for the unusual attributes of this particular allele, i.e., its high frequency of postmeiotic segregation when heterozygous, its marker effects in recombination, and its nonrevertible character (6).

Ultimately, the ade8-18 sequence may be compared to ade8-14, an adjacent, effectively inseparable ochre mutant known to revert and which nonetheless exhibits equivalent PMS patterns when heterozygous. However, ade8-14 does not exhibit the pronounced marker effect of stimulating recombination rates with alleles to the left or right as is the case with ade8-18 (5). Clearly, DNA sequence data can be expected to illuminate our insight concerning recombination at the intragenic level. Currently, we can orient the cloned fragment relative to Esposito's fine structure map shown in Fig. 3. The ade8-10 site falls between the restriction sites EcoRl and SalI and the ade8-18 site is localized on the 1750 bp segment bounded by SalI and BamHI. Determined also is the orientation of the 2.5kb subclone. The ade8-10 allele located at the low end of the conversion polarity gradient is near the EcoRI site and the ade8-18 allele distinguished by high conversion and high PMS rates (17) falls on the opposite side of the SalI site. The availability of two KpnI sites and single sites for HpaI, XhoI, and BglII provide additional subcloning opportunities.

A similar combined genetic-molecular approach is projected for the specific mutant allele arg4-16. This mutant also displays an exceptionally high meiotic conversion rate and a high proportion of these events, 30%, are postmeiotic segregations (35). The isolation and cloning of the ARG4 locus was definitively established earlier by others and we have obtained an

| Bam HI | | | Eco RI | Sal I | Xho I | | Bgl II | Bam HI |
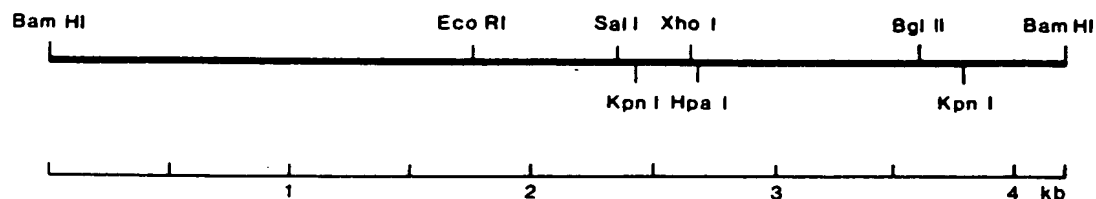
Fig. 2.   Restriction map of the 4 kb fragment.

MEIOTIC

⊢——⊣ 70 prototrophs/10^5 asci

X-RAY

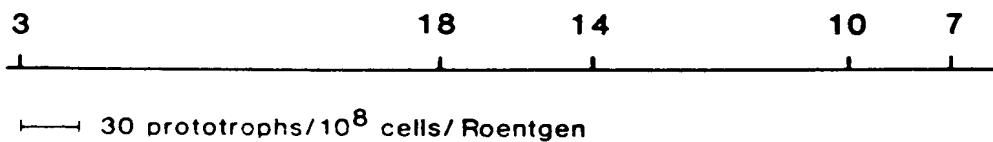⊢——⊣ 30 prototrophs/10^8 cells/Roentgen
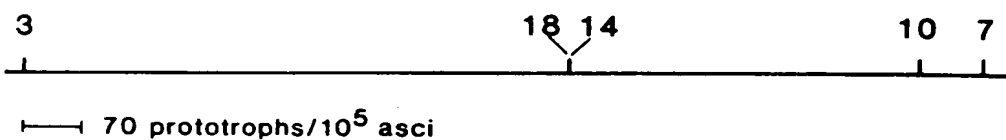
Fig. 3.   Genetic fine structure map of Adenine8, after Esposito (1967).

independent clone.   Experiments with the ARG4 clone will be discussed else-
where.

ADE8 integration—a visual screen

When the YRp17 plasmids carrying either the 2.5 or 4.0 kb cloned frag-
ments are employed to transform spheroplasts of a normal MATa/MATα diploid
strain homozygous for the markers ade2, ade8-18, ura3 and trp1, selection for
positive URA3 and TRP1 function is achieved by plating the protoplast-plasmid
mixture in soft agar on normal solidified media deficient for tryptophane and
uracil.   Such transformants display behavior expected of cells containing an
autonomously replicating plasmid. When the selective pressure is relaxed, by
growing transformed cells on a rich medium such as YEPD, a large proportion of
the daughter cells, and their subsequent progeny, lack the plasmid and conse-
quently display a trp1 ura3 phenotype. These observations are fully con-
sistent with those made earlier (32,33).

Transformed ade2, ade8-18 cells, i.e., containing the autonomously repli-
cating plasmid described above, appear white rather than red on YEPD and other
media. Since ADE8 function is undoubtedly provided by the plasmid, as indi-
cated by transformation of ade8-18 ADE2 strains to prototrophs, it is
appropriate to inquire concerning the underlying causes. Factors contributing
to this altered phenotypic expression are as follows:

1. Cells that have lost the plasmid, or failed to acquire it, are ade2
ade8-18 genotypically.   Such cells are devoid of red pigment since the
metabolic block imposed by ade8-18 occurs prior to the ade2 block in the
de novo biosynthetic pathway.

2. White cells typically outgrow red cells.

3. Transformed cells may excrete uracil and tryptophane and these
nutrilites are utilized for growth by ade2, ade8-18 white cells.

Transformed cells are, at best, faint pink on trp ura media and
moderately pink on trp ura ade media. Such white or pink patches often
display numerous clearly defined sectors which are dark red by comparison to
the adjacent pink/white background and exhibit a pigmentation level charac-
teristic of ade2 ADE8 cells. Subsequent tetrad analysis indicates that each
red sector represents a single, independent integration event. Accordingly,
the white-red or red-white shift, here designated as the Roman effect, in
honor of Prof. H. Roman who first exploited the red-white system in yeast
genetics (29,30), serves as a visual screen for the detection and ready isola-
tion of homologous recombination events in which a plasmid is incorporated
into the recipient's genome. Furthermore, the red-white Roman effect forms
the basis for quantifying intrachromosomal gene conversion rates as well as
plasmid excision rates.

The system's clarity and in its objectivity make it unusually adaptable
as a novel means for identifying and characterizing specialized DNA sequences
of yeast, or other organisms, that mediate, regulate or control general as
well as site specific recombination.   Even greater resolving power may be
introduced by coupling our systems to other recombinant DNA constructions that
are highly selective.   In this manner, only excision products would survive.

Among these, the proportions of red and white colonies could be determined with high speed counting devices. Accordingly, integration and excision might be amenable to a highly detailed molecular and genetic characterization.

Thirty two (32) independent red sectors were isolated and sporulated. Ascus dissection and tetrad analyses were performed. Twenty-eight red-sectored clones were heterozygous for a single integration event--ADE8 cosegregated with URA3, TRP1 in the 4:4 ratio expected for simple Mendelian behavior. Three integrants were apparently homozygous. These might represent two simultaneous integration events, mitotic co-conversions, or mitotic recombination between the centromere and the site of integration. Finally, a single sector was simply heterozygous for ADE8/ade8-18. Here, we suppose that an intrachromosomal excision event resulted in the loss of the segment containing URA3, TRP1 ade8-18. Alternatively, it might more likely represent an informational transfer from the plasmid to the chromosomal site. Transfers in the opposite direction may occur also. This isolate, Bam5, serves as our standard conventional heterozygote. It displays a basic conversion frequency of 10% among 621 fully analyzed tetrads. Of the 63 aberrant segregations, 40 or 63.5% displayed postmeiotic segregations, detected as sectored ascosporal colonies by means of our standard plate-dissection-replica-plating procedure.

Site of integration

Red, URA3, TRP1, ADE8 segregants from each of twenty-one integrants involving the cloned 4kb fragment, were crossed to a single tester strain displayed in Table 1. Single zygotes were isolated, grown, sporulated, and subjected to dissections and tetrad analysis. Table 1 presents the diagnostic screen for identifying the site of integration. Of the twenty-one integrant hybrids analyzed in this manner, 18 displayed segregations consistent with integration at or near the ade8-18 site on chromosome IV. In addition, three integrations occurred close to the centromere at or near trp1. To a first approximation, it appears as though the frequency of integration at a given site is proportional to the physical length of the homologous segment carried in the plasmid. Thus, the TRP1 segment and the Bam H1 fragment containing ADE8 within the YRp17 vector are respectively 1.43 and 4.0 kb; or a ratio of about 3. Also, it may be noted that when the ADE8 segment is reduced to about 2.5 kb, a significantly higher proportion of the integration events (about 50%) occur at trp1 compared to ade8-18. These findings suggest that the probability of localized effective mitotic pairing, as might be required for integration events involving mitotic recombination between homologous sequences, increases as a simple function of the physical length of the interactive sequences. Finally, as regards homologous sequence integration events, the three apparent homozygotes, about 10%, suggest that integration itself may be recombinogenic. Experiments specifically designed to test this notion in a critical manner are warranted.

Rates of Mitotic Intrachromosomal Excision

Events leading to the excision of YRp17 plasmid sequences containing ADE8 inserts can be studied quantitatively with respect to the actual recombination rate at which the integrated plasmid sequence is removed from the genome. Mitotic excision rates are readily estimated from protocols based on the method of the median (7,9). In our studies, eleven independent haploid colonies, each containing about 1.5×10^{7} cells, were assayed via dilution-

TABLE 1. Diagnostic segregation and genetic screen for identifying site of integration

	trp1	TRP4	ade8-18 URA3 TRP1 ADE8	RNA3	ura3	ade2
Integrant	o———o		o————————o		o————o	o
×						
Tester	TRP1	trp4	ade8-14	rna3	URA3	ade2
	o———o		o————————o		o————o	o

Tetrad segregation on synthetic complete medium deficient for:

Site of Integration	tryptophane	adenine			uracil
ura3	$2^+:2^-$	18	18	18	
	$1^+:3^-$	18	14 or	14	$4^+:0^-$
	$0^+:4^-$	+	or +	+	
		+	+	+	
trp1	$2^+:2^-$	14	18	18	$2^+:2^-$
		14	18 & or	14	$3^+:1^-$
		+	+	+	$4^+:0^-$
		+	+	+	
ade8	$2^+:2^-$	14			$2^+:2^-$
	$1^+:3^-$	14			$3^+:1^-$
	$0^+:4^-$	+			$4^+:0^-$
		+			

plating on non-selective media and subsequent replica-transfers to diagnostic media. Each colony arose from a single cell isolated by micromanipulation. Incubations were at 30° for three days. Three quantities are ascertained: a) Numbers of cells in each of the eleven parental colonies, b) Proportions of cells within each colony that yield trp ura colonies and c) Proportion of the auxotrophic colonies that are red (ADE8) or white (ade8-18). White colonies are classified as ade8-18 only on the basis of appropriate complementation tests. Such estimates allow us to compute a recombination rate for excision in terms of a probability per cell division.

Excision rate experiments were conducted wth non-tandem duplication haploid strains derived from the meiotic analyses of integrants containing ADE8 fragment of 4.0, 2.5 and 1.7 kb in length. The integrations occurred at ade8-18, ade8-10 or trpl.

Taken collectively, the data obtained are consistent with the following simple model: We assume that the mitotic excision rate is determined primarily by the sequence homology length between the recipient and inserted sequences in a given non-tandem duplication. Exchanges leading to excision are assumed to occur randomly along the homologous segment. Accordingly, with a particular integration array e.g., ADE8-URA3-TRP1-ade8-x or ade8-x-URA3-TRP1-ADE8), the proportion of ade8-x-ura3-trpl to ADE8-ura3-trpl excision products would be determined by the position of the ade8-x mutant site within the region of homology.

When the recipient genome contained the mutant allele ade8-18, the rate leading to white (ade8-18) excision products is about four times the rate to red, i.e. 7.3×10^{-5}/division vs 1.9×10^{-5}/division. A representative set of data is displayed as Table 2. However, when the recipient genome contained the mutant ade8-10 in place of ade8-18, the corresponding rates to white and red excision products were essentially equal—i.e. 3×10^{-5}/division vs 2.5×10^{-5}/division. We may note that ade8-10 is located within the BamHI-SalI section. Thus, the ade8-10 site divides the 4 kb insert nearly equally, whereas the ade8-18 mutant divides the same fragment disproportionately.

Given an integration event of the type ....ade8-URA3-TRP1-ADE8....., a crossover at a or b would generate red or white excision products respectively as shown in Fig. 4. The ratio of red to white excision products is approximately equal to the ratio of the sequence homology lengths in region b compared to region a. Illustrative data for the 4 kb fragment integrated into recipients carrying ade8-18 and ade8-10 are given in Tables 2 and 3.

In addition to varying the position of the mutant site achieved by utilizing different alleles, we may also vary the extent of homology by choosing appropriate subclones of the original 4 kb BamHI fragment. As given in Fig. 2, an EcoRl site allows us to isolate a YRpl7 plasmid containing a 2.5kb insert that confers ADE8 function. Non-tandem duplications derived from this vector were studied with respect to spontaneous mitotic excision. Integrations into haploid recipient strains carrying ade8-18 and ade8-10 were analyzed, and the data is presented in Table 4. In this instance, the ade8-10 site divides the 2.5 kb insert into nearly equal segments. Thus, our model predicts that the ratio of (rate to reds)/(rate to whites) would be unity. The overall total excision rate for this smaller fragment would be little more than half the rate obtained with the 4 kb fragment. Also, reduced rate

TABLE 2. Plasmid Excision Rate: 4kb Insert Integrated at ade8-18

Colony	Units/Colony	Units Plated	trp⁻, ura⁻ White	trp⁻, ura⁻ Red	Rate (excisions/cell/div) White	Rate (excisions/cell/div) Red
1	1.43×10^7	2808	2	2	4.3×10^{-5}	4.3×10^{-5}
2	2.46×10^7	2357	15	1	3.8×10^{-4}	2.5×10^{-5}
3	1.44×10^7	1870	2	0	6.7×10^{-5}	—
4	1.40×10^7	1913	0	0	—	—
5	1.14×10^7	2341	3	4	8.0×10^{-5}	1.1×10^{-4}
6	1.41×10^7	2176	2	0	5.6×10^{-5}	—
7	1.12×10^7	2153	4	0	1.2×10^{-4}	—
8	1.40×10^7	2226	3	0	6.7×10^{-5}	—
9	1.51×10^7	2084	5	0	1.5×10^{-4}	—
10	1.49×10^7	2236	50	0	1.3×10^{-3}	—
11	1.38×10^7	2528	3	1	7.3×10^{-5}	2.4×10^{-5}
Total	1.62×10^8	25092	89	8		

Median Rate to ade8-18, trp⁻, ura⁻ from colony #11: 7.3×10^{-5} excisions/cell/division

Mean Rate to ADE8⁺, trp⁻, ura⁻ from total data: 1.9×10^{-5} excisions/cell/division

Calculated from Rate= $((.4343) \times (\text{freq. trp}^-, \text{ura}^-))/\log_{10}(N_{\text{final cells}})$

TABLE 3.   Plasmid Excision Rates

Size of Insert	Integration Site	Rate* to	
		mutant	wild
4 kb	ade8-18	7.3×10^{-5}	1.9×10^{-5}
4 kb	ade8-10	3.0×10^{-5}	2.5×10^{-5}
4 kb	trp1-1	7.6×10^{-5}	8.1×10^{-5}
2.5 kb	ade8-18	4.1×10^{-5}	3.4×10^{-6}

*Rate = excisions/cell/division
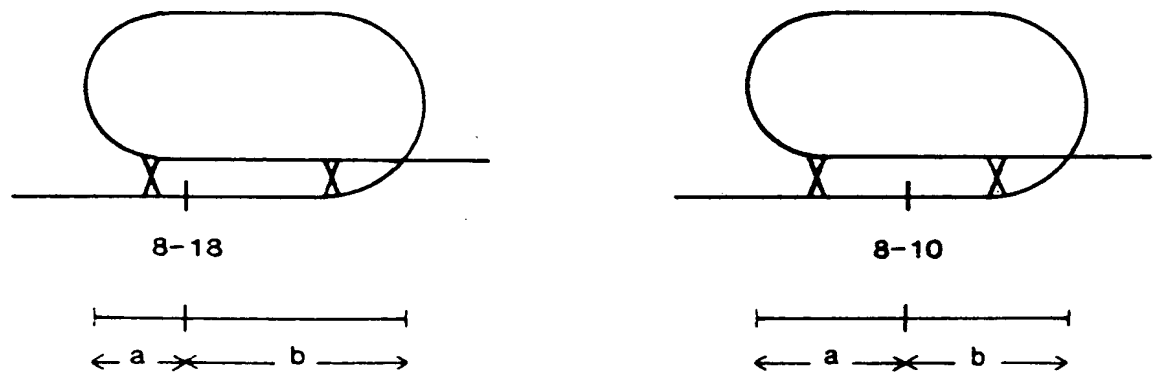
Fig. 4.   Intrachromosomal excision events relative to mutant sites in ADE8.

TABLE 4. Gene Conversion and PMS in Hybrids with 2,3, and 4 Adenine8 Sequences

Sequence Array	Strain	Total Tetrads	$4^+:4^-$	$6^+:2^-$	$2^+:6^-$	$5^+:3^-$	$3^+:5^-$	$7^+:1^-$ & ab. $6^+:2^-$	%BCF*	%PMS/BCF
+ / −	Bam 5	622	560	15	7	21	18	1	10.0	64
− / + / −	Bam 9	515	493	8	3	7	4	0	4.3	48
− / −	JK101	582	547	6	9	5	15	0	6.4	57
− / −	TC140	554	—	5	15	5	8	0	6.0	39
−	Bam 32	640	583	33	0	24	0	2	9.3	44
− / −	TC136	820	692	110	0	18	0	0	15.6	14

Sequence Array	Strain	Total Tetrads	$8^+:0^-$	$6^+:2^-$	$7^+:1^-$				%BCF*	%PMS/BCF
+ / − / + / −	TC148	174	168	2	4				3.4	67

*BCF, the Basic Conversion Frequency, is defined as $((6^+:2^- + 2^+:6^- + 5^+:3^- + 3^+:5^- + 3^+:5^-)/\text{Total Tetrads}) \times 100\%$

would arise at the expense of ade8 excision products, since the smaller fragment was generated by removing 1.5 kb of DNA from the b region of the original 4kb segment. To date, all of our data on mitotic excision in non-tandem duplications is consistent with the simple model presented above.

Finally, we may inquire about mitotic excision s as a function of where the integration site is located within the genome. Three integration events were detected in the vicinity of trpl, a centromere-proximal marker whose extent of sequence homology corresponds to a 1.4 kb TRPl fragment carried in the YRpl7 vector. On the meiotic map (26,27) trpl is located about 0.5 cM from the centromere while ade8 is removed about 150cM distally on the same arm of chromosome IV. A single integrant at trpl was analyzed in detail. The non-tandem duplication has the structure cen- trpl-ADE8-URA3-TRPl.......ade8-18... ..ade2. The total excision rate approximates that observed for excision of the same plasmid when it is incorporated into the genome at ade8-18. Also, the TRPl ura3 ade8-18 excisions were equal to the trpl ura3 ade8-18 excisions. Thus, the rates of spontaneous mitotic excision for plasmid sequences introduced into the chromosome by homologous recombination are not markedly affected by proximity to a genomic centromere. In summary, it is clear that non-tandem duplications that arise by the integration of cloning vehicles into the genome provide us with a sensitive device for estimating several important parameters of recombination. Furthermore, as presently constituted or coupled to selective DNA construction, the system could serve to identify and isolate DNA sequences that regulate mitotic recombination. As yet, we have not varied the distance between the duplicated sequences, though this is readily achieved by recombinant DNA in vitro procedures.

Meiotic gene conversion and postmeiotic segregation in non-tandem duplications.

Most of the independent integration events (28/32) were heterozygous for the plasmid markers, i.e. URA3, TRPl and ADE8 (see Fig. 1). 515 complete four-spored tetrads from an integrant at ade8-18 were analyzed. This culture is listed as Bam9. In all, twenty-two conversion-like events were detected via the plate dissection-replica transfer method. This reflects a basic conversion frequency of 4.3%--a value equal to about half that of the standard isogenic heterozyote Bam5, where 622 analyzed tetrads yielded a basic conversion frequency of 10%. However, the % PMS/BCF values, 48% and 64%, for Bam9 and Bam5 respectively, are essentially equivalent. Thus, we presume that the presence of the integrated plasmid, yielding a non-tandem duplication, reduces the frequency of heterozygous pairings. Pairings are of two types i.e., +/- or-/- and if these occur with equal liklihood and the repair parameter (50%) is unchanged, our predicted value for %BCF and % PMS/BCF would concur with those observed (see Table 4). Clearly a, a novel prediction is suggested by these findings. If the non-tandem duplications were iterated as +----- we would predict a reduction in % BCF proportional to the number of iterated ade8-18 alleles, but with no change in the % PMS/BCF ratio. Recombinant DNA constructions of this sort have been reported (23).

The availability of non-tandem duplication, such as ADE8-URA3-TRP1-ade8-18 as well as the corresponding homosequential forms ADE8-URA3- TRP1-ADE8 and ade8-18-URA3-TRP1-ade8-18 obtained via intrachromosomal information transfer, allow us to generate fifteen unique hybrids that carry two, three or four ade8 alleles per diploid. Among these, ten contain dissimilar homologous

chromosomes.   Data   pertaining   to   five   situations is given in Table 4.   We examine first DK101, a diploid with the following genotype: ade8-18 URA3 TRP1 ade8-18/ADE8.

The meiotic behavior of this hybrid, containing two alleles and one allele on respective homologues, may be contrasted with Bam5. This isogenic control yielded data that is statistically homogeneous with our previous analysis of 15,480 tetrads (16,17). Both samples display a slight but significant disparity relative to the strand on which intragenic recombination is initiated. This may be expressed by the dissymmetry coefficient of $(6^+:2^- + 5^+:3^-)/(2^+:6^- + 3^+:5^-)$ which is 1.36 in the 15,480 tetrad sample and 1.5 in Bam5. In DK101 the dissymmetry coefficient falls to 0.46 i.e., 11/24. How can these findings be rationalized in terms of a simple testable molecular model?

We may presume that the dissymmetry coefficients characterizing the control data reflect a higher liklihood of conversional initiations involving the strand bearing the wild type allele as given in the Aviemore model (24). We may note that in the total control data, there are 396 and 312 PMS events of the $5^+:3^-$ and $3^+:5^-$ varieties respectively. Taken at face value, these data indicate that the disparity arises prior to the occurrence of correctional repair on the heteroduplex. It is only subsequent to this repair that gene conversion or restoration are established. Given the above, we emphasize that one strand in DK101 bears two ade8-18 sequences while the homologue bears only a single ADE8 sequence. Thus, the observed dissymmetry coefficients can be accounted for as follows: Since DK101 contains two mutant alleles on one strand and a wild type allele on the other, we may assign to these initiation likelihood values derived from the control data, i.e. 0.423 x 2 and 0.527 respectively. Normalizing these to unity, we obtain 0.595 for the strand with two ade8-18 alleles and 0.405 for the wild type strand. Thus, with these derived initiation values, we predict 0.405 x 35 = 14 events of the $6^+:2^- + 3^+:5^-$ type and 0.595 x 35 = 21 events of the $2^+:6^- + 3^+:5^-$ type. The actual number of observed events corresponding to these classes are 11 and 24 — an acceptable fit between the observed data and hypothesis.   In summary, each sequence retains its normal properties in such non-tandem duplications.

A similar analysis applies to TC140. Here, the two homologues contain ADE8-URA3-TRP1-ade8-18 and ade8-18-URA3 TRP1-ade8-18. If misalignment of homologous sequences did not occur, or occurred with a negligible frequency, this diploid should exhibit a dissymmetry coefficient equivalent to the standard value 1.36. The observed value 0.43 (10/23) is significantly lower than the predicted value. Now, if we assume that misalignment at meiotic prophase may occur, we visualize that three different synaptic configurations might occur with equal probability or 0.33; these patterns are:

ADE8 - URA3 - TRP1 - ade8-18
ade8-18-URA3 - TRP1 - ade8-18

```
                      ADE8  -  URA3  -  TRP1  -  ade8-18
ade8-18 - URA3 - TRP1 - ade8-18

ADE8  -  URA3  -  TRP1  -  ade8-18
                      ade8-18  -  URA3  -  TRP1  -  ade8-18
```

It may be noted that the first two configurations can readily yield gene conversions and postmeiotic segregations, while the last configuration can yield only a homoduplex at the paired ade8-18 sites. Alternatively, the unpaired ADE8 segment might behave as wild type sequence heterozygous for a deletion. In the latter instance, no postmeiotic segregations would be generated. Thus, overall we predict a reduction of the basic conversion frequency by a fraction of about 1/3 or to 6.7%-- a value in agreement with the observed 6.0%. Again, it may be noted that in this hybrid, containing a single heterozygous site within otherwise identical non-tandem duplications in each homologue, the respective ade8 sequences retain their individual properties.

In the culture designated Bam32, with 642 total tetrads analyzed, we may observe a situation that is essentially the reverse of DK101. Here, an isosequential non-tandem duplication for the wild type ADE8 sequences is heterozygous for a single copy of the mutant allele ade8-18, i.e., $\frac{ADE8\ URA3\ TRP1}{ADE8/ade8-18}$ Several features of the data may be noted. First the $2^-:6^+$ and $3^+:5^-$ categories are conspicuously absent. Moreover, the $6^+:2^-$ and $5^+:3^-$ classes occur with approximately equal frequency. The wide ratio class contains a single ab $6^+:2^-$ and one $7_+:1_-$ or events taken to represent two independent heteroduplexes. In the ab $6^+:2^-$, two sectored ascosporal colonies accompany two wild type colonies; in the $7^+:1^-$ a single sectored ascosporal colony accompanies three corresponding wild type colonies. Thus, the ab$6^+:2^-$ ascus may reflect two separate events that involve all four chromatids and where the heteroduplexes span a single ade8-18 site and these are passed uncorrected into the functional ascospores. Likewise, the $7^+:1^-$ segregation mirrors two equivalent occurrences and presumably only one of the heteroduplexes is repaired in the direction of wild type. Adding the respective $6_+:2_-$ and $5^+:3_-$ segregations generated by the wide ratio asci to the observed $6^+:2^-$ and $5^+:3^-$ clones, we obtain 34 and 27 events respectively or a %PMS/BCF of 44.3%, a value roughly comparable to the corresponding ratio in our control sample—52%. The absence of apparent $2^+:6^-$ and $3^+:5^-$ segregation asci can be accounted for by simply assuming that homologous pairing and heteroduplex formation spanning the ade8-18 site, with or without correctional repair will be associated with an unaltered ADE8 sequence in cis array.

Accordingly, potential conversions or PMS segregations resulting from events initiating on the ade8-18 strand remain cryptic. Masked by the ADE8 on the same strand, such events therefore appear as ordinary $4^+:4^-$ segregations.

We may proceed to calculate the expected number of cryptic events and subsequently compare this theoretical value with a value determined from an experimental procedure described below. Using the same rationale for normalization as in DK101 (0.577+0.577+.423=1.577), we obtain 1.154/1.577=0.732 and 0.423/1.577=0.268-- the respective initiation probabilities for the corresponding parental strands ADE8 URA3 TRP1 ADE8 and ade8-18. Because 61 events were observed and since these represent only 73.2% of the total, the actual number of events should be corrected to 83.3. Accordingly, 26.8% of the total events should have initiated from the mutant ade8-18 strand and yielded 22.3 cryptic events to be found among the 583 normal $4^+:4^-$ segregations.

Now, we may consider the experimental protocol for detecting cryptic events. A total of 167 normal $4^+:4^-$ meiotic segregations from Bam 32 were

tested as follows: the red segregant ascosporal clones were patched to YEP master plates. Individual white colonies from each patch were isolated, purified and tested for tryptophane and uracil requirements as well as complementation by crossing with an ade8-18 tester strain. The tested cultures might be ADE8 -URA3-TRP1-ADE8 , or normal. Alternatively, they might be ade8-18 URA3 - TRP1-ADE8 i.e., conversions, or ade8-18/ADE8-URA3-TRP1-ADE8 i.e. a PMS. In either case, the conversions and the PMS events will generate white colonies that are demonstrably ade8-18 genotypically. Such derivatives might arise as a consequence of intrachromosomal gene conversion with or without associated exchange. We examined 334 red ascosporal colonies from 167 complete asci. Twenty four colonies, each representing a single tetrad, yielded the expected white derivatives. However, in all but two instances, these were URA3 TRP1 and ADE8. Hence, they mirror spontaneous mutantional alterations at unrelated loci that impose blocks in the de novo adenine biosynthetic pathway prior to ade2—another manifestation of the Roman Effect. The two remaining instances were ade8-18-ura3-trp1 and hence reflect the cryptic gene conversion or PMS along with the excision of the original plasmid sequence either subsequently or simultaneously. Finally, we may compare our observed number of two cryptic events with the number expected or 6.3 events (167/583 x 22). We may conclude that cryptic events do in fact occur and these arise in proportions similar to those given above. Hence, the basic conversion frequency of 9.3% has been underestimated by a failure to include twenty two cryptic events. The adjusted value is 12.9% BCF.

The possibilities for the analysis of synaptic misalignments and therefore unequal meiotic crossing-over is especially favored in the diploid TC 136. Here, two different isosequential non-tandem duplications are combined as follows: ADE8-URA3-TRP1-ADE8/ade8-18-URA3-TRP1-ade8-18.

One homologue carries two ADE8 arrays and the other carries two ade8-18 arrays. Otherwise, the homologues are congenic, though each was derived by an intrachromosomal gene conversion of separate integration events.

In all, 820 complete tetrads were analyzed (Table 4). The apparent %BCF was 15.6% with 110 segregations of the 6^+:2 variety and 18 tetrads displayed 5^+:3 patterns. However, 2^+:6 and 3^+:5 asci were absent. What rationale will account for the marked disparity observed in these data as well as the rather high %BCF value. A priori, we might consider that each heterozyous site of the non-tandem duplication might behave independently of the remaining site. Conversions of mutant to wild at either site would yield 6^+:2 tetrads and uncorrected heteroduplexes formed by initiations on the wild type strand would generate 5^+:3 asci. In contrast, 2^+:6 tetrads would require simultaneous conversions of wild to mutant at both sites and PMS events with initiations on the ade8-18 stand would remain cryptic. To the above we must add the contribution of reciprocal exchange between the duplicated sequences. Such events would appear as 6^+:2 segregations superficially, but crypticity tests would reveal that two ascosporal clones were heterosequential. In contrast 6^+:2 tetrads arising from informational transfer alone would contain a single heterosequential spore clone. Further speculation in the absence of adequate crypticity analysis is not warranted. Considered together, the factors discussed above provide a basis for the observed disparity.

A qualitative statement bearing on the question of misalignment during meiotic prophase may be obtained by considering the 174 unselected tetrads for

hybrid TC148. This diploid is homozygous for a heterosequential non-tandem duplication. As displayed in Table 4, six aberrant tetrads were identified; two were $6^+:2^-$ and four were $7^+:1^-$. The $6^+:2^-$ asci may have originated as intrachromosomal gene conversions of the wild to the mutant sequence; or they may reflect misalignments associated with a similar conversional direction. The $7^+:1^-$ segregations are all taken to represent PMS events—i.e. misalignments accompanied by information transfer from the mutant sequence to the wild type sequence without correction. Thus, some of the meioses display evidence that is indicative of misalignments or intrachromsomal events.

In conclusion, it is apparent that these studies must be regarded as only partial. Required for a total analysis is a more extensive study of crypticity coupled with a rigorous molecular approach via DNA-DNA hybridization and sequence determinations of the various wild type and mutant alleles.

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March 7, 1996

VIA FEDERAL EXPRESS

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Dear Dale:

Enclosed please find the following documents to be hand delivered to the U.S. Patent and Trademark Office (Group Art Unit:1812), with a courtesy copy to the examiner:

1. Reply and Amendment
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Please also ensure that the attached postcard is date stamped "received" and returned to us. March 8, 1996 is a 6 month bar date so it must be filed on March 8, 1996. If you have any questions with regard to the foregoing, please do not hesitate to contact us. Thank you for your assistance with this matter.

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ORIGINAL PUBLICATIONS:

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conference, Amyloid and other abnormal protein assembly processes, August, 1995.

AFFILIATIONS:
American Chemical Society
Society for Neuroscience
International Brain Research Organization
American Association for the Advancement of Science
Foundation for Biomedical Research
American Peptide Society
International Neuropeptide Society
Boston Area Neuroscience Group

COMPETITIVE SUPPORT:
Science and Engineering Research Council, UK (1981-82)
National Science Foundation (1982)
Muscular Dystrophy Association (1983-84)
North Atlantic Treaty Organization (1982)
National Institute of Neurological Disorders & Stroke (1985-1994)
Milton Fund of Harvard University (1989) (1994)
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TRAINING PROGRAMS (NIH) at Harvard Medical School:
Pharmacological Sciences (Associate)
Endocrinology (Associate, Executive Committee)
Neuroscience (Associate, Admissions, Appointments, Steering Committee)
Developmental Neurology (Associate)
Molecular Biophysics (Associate)
Neurological Sciences Academic Development (Associate)
Biological Sciences in Public Health (Associate)

LOCAL COMMITTEES:
Dr. Maggio has served on and chaired a wide range of departmental, program, medical school, and university committees, such as: Faculty Search, Admissions, Facilities, Thesis Advisory, Qualifying/Preliminary Examination, Prize, Curriculum, Thesis Defense, Course Planning, Steering, Executive, Criteria, Etc. *See Service.*

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NIH Study Sections (ad hoc, Reviewers Reserve):
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   Small Business Innovation Research (SSS-7/E, 3/88)
   AIDS and Related Research (ARR-5, 12/88)
   Neurological Sciences (NLS-1, 6/90, 10/90, 6/91, 10/91, 10/92, 10/93,
      10/94)
   Neurology (NEUB-1, 6/95)

NSF Applications (ad hoc)

Journals (ad hoc):
   Am. J. Pathol., Am. J. Physiol., Anal. Biochem., Anesthesiology,
   Biochemistry, Biochem. Biophys. Acta, Brain Res., Cancer Res., FEBS
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   Med., J. Neurochem., J. Neuroimmunol., J. Neurosci., Lab. Invest.,
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   Sci. USA, Protein Sci., Regul. Peptides, Trends Neurosci.

BRIEF PROFESSIONAL BIOGRAPHY:
Dr. Maggio's graduate work included research on noncovalent interactions, organic synthesis, reaction mechanisms, biological control, NMR spectroscopy, and biochemistry under the mentorship of Jean-Marie Lehn, Robert B. Woodward, and (principally) Konrad E. Bloch. He received the Ph.D. in Organic Chemistry from Harvard University in 1981. His postdoctoral research on various aspects of neuropeptides and neuropeptide receptors was carried out at the Medical Research Council and the University of Cambridge, UK, with Leslie L. Iversen and Dudley H. Williams; and later at Yale University School of Medicine with Robert H. Roth. He joined the faculty of Harvard Medical School in 1985, and is presently Associate Professor of Biological Chemistry and Molecular Pharmacology.

RESEARCH INTERESTS

The bioactive peptides are the largest and least understood class of intercellular messengers, carrying out a diverse set of functions in a wide variety of systems. Understanding bioactive peptides and their receptors, in the nervous system and elsewhere, is the general research goal in our group.

One system of interest is the tachykinin (substance P) family of peptides and receptors, which are involved in transmission of primary afferents and thus in pain and neurogenic inflammation. As the primary structures of both the ligands and their receptors are known, an excellent model system for peptide-protein interactions in signalling is available. Recently we have identified through photoaffinity labelling which regions of the peptide substance P interact with which regions of its G-protein-coupled receptor, a protein whose expression is upregulated a thousand-fold in some inflammatory diseases. Radioactive, fluorescent, and antibody probes of these receptors allow studies of desensitization and internalization *in vivo* and *in vitro*.

Another system under investigation is the process of amyloid formation in Alzheimer's disease (AD) and other amyloidoses. The characteristic lesion of AD is brain senile plaques formed mainly of the human amyloid peptide Aß, a ≈40-mer which occurs naturally in normal as well as AD brain. By reconstituting plaque growth (deposition of Aß at physiological concentrations onto authentic plaques) *in vitro*, we can characterize the process and identify conditions and components which enhance or inhibit its kinetics. Structure/activity studies have identified amino acids critical for amyloid deposition and active peptide analogues suitable for high resolution structure determination by nuclear magnetic resonance spectroscopy. The latter studies have further identified conformational elements essential to plaque deposition.

Another interest is the characterization of novel bioactive peptides from natural sources. A particularly rich source is the skin venom of certain neotropical frogs. The peptides found here include antibiotics and toxins as well as close analogs of discovered and yet undiscovered mammalian neuropeptides.

REFERENCES available on request.

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Miklos Bodanszky

Peptide Chemistry

A Practical Textbook

Second, Revised Edition

With 2 Figures and 5 Tables

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To the memory of
my brother Dr. S. Bodanszky

Professor Dr. Miklos Bodanszky
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should be applied in an excess that provides for a concentration of at least 10^{-1} M throughout the coupling reaction. This measure (the "principle of excess") counteracts the decrease in rate which necessarily occurs in bimolecular reactions as the concentration of the reactants decreases. Therefore the extent of unimolecular side reactions, in which the rate is independent of concentration, can be markedly reduced. An excess of acylating agent also helps to achieve complete acylation of the amine-component and prevents thereby the formation of "deletion sequences", peptides from which one amino acid residue is missing.

A further concentration related problem has to be mentioned here. Following deprotection by acidolysis the regenerated amine is isolated as a salt of the acid used for cleavage. In the subsequent acylation step, however, the free amine is needed. Deprotonation with the help of ion exchangers can be applied or, in solid phase peptide synthesis (Chapter X) treatment with a tertiary amine and removal of the trialkylammonium salts by washing. In syntheses carried out in solution the general practice is to "liberate" the amine-component from its salt by adding an equimolar amount of a tertiary amine (triethylamine, diisopropylethylamine, N-methylmorpholine or N-ethylpiperidine) to the reaction mixture prior to coupling. It is obvious, however, that often only an equilibrium

$$R-NH_3^+ + R_3'N \rightleftharpoons R-NH_2 + R_3'NH^+$$

can be established. While it is true that during acylation of the amino group this equilibrium is gradually shifted to the right, at any given time the concentration of the amine-component is lower than it would be if applied in completely deprotonated form. A notable exception is created by the insolubility of tertiary ammonium salts in certain solvents. For instance triethylamine hydrochloride, being practically insoluble in ethyl acetate, separates from the reaction mixture (if ethyl acetate is used as solvent for coupling) and shifts the equilibrium in the desired direction. The presence of tertiary amines is usually unfavorable during coupling: they can initiate side reactions through proton abstraction. These reactions can be suppressed by the addition of certain weak acids, for instance 2,4-dinitrophenol or pentachlorophenol, which show a distinct affinity for tertiary amines. They do not protonate the amine-component firmly enough to prevent its acylation. Moreover, the application of tertiary amines can be avoided by selecting highly acid sensitive amine-blocking groups and removing them with suitable weak acids, such as 1-hydroxybenzotriazole or tetrazole. The resulting salts are readily acylated, even with moderately reactive esters, without the addition of a tertiary base.
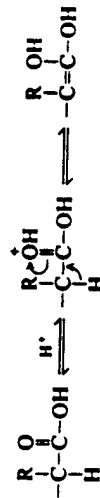
Additional Source

Bodanszky, M., Martinez, J.: Side Reactions in Peptide Synthesis, in The Peptides, vol. 5, Gross, E., Meienhofer, J. eds., pp. 111–216, New York, Academic Press 1983

VIII. Racemization
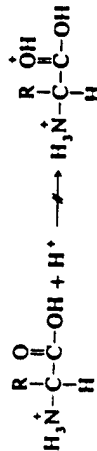
With the exception of glycine, in all amino acids that are constituents of proteins, the α-carbon atom is chiral. In threonine and isoleucine a chiral center is present in the side chain as well. In order to secure the target peptide in homogeneous form it is absolutely essential to start from enantiomerically pure amino acids and to insist on conservation of chiral homogeneity throughout the various operations of synthesis. Otherwise, instead of a single product, a mixture of stereoisomers will be obtained. Their number in a peptide with n chiral centers is 2^n. Accordingly, if racemization is not prevented, even in the synthesis of a moderately large peptide a complex mixture will be produced and separation of the desired material from a multitude of similar compounds might turn out to be an at least arduous and sometimes overwhelming task. Therefore, the importance of racemization studies and of the measures that must be taken for the prevention of any loss in chiral purity can not be overemphasized. In fact, "strategies" of peptide synthesis, that is general planning of schemes for syntheses (Chapter IX) are dictated primarily by considerations concerning conservation of chiral homogeneity.
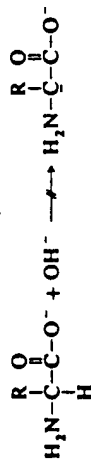
A. Mechanism of Racemization

With respect to chiral stability amino acids are fairly insensitive to acids and bases. Racemization via enolization of carboxylic acids in acidic solutions involves protonation of the carbonyl oxygen

$$R-\overset{O}{\underset{H}{C}}-C-OH + H^+ \rightleftharpoons R-\overset{\overset{+}{O}H}{\underset{H}{C}}-C-OH \rightleftharpoons -\underset{H}{C}-C-OH \rightleftharpoons -C=C-OH$$

yet, the presence of a nearby positively charged nitrogen atom hinders the formation of the second cation:

$$H_3\overset{+}{N}-\overset{O}{\underset{H}{C}}-C-OH + H^+ \longleftrightarrow H_3\overset{+}{N}-\overset{\overset{+}{O}H}{\underset{H}{C}}-C-OH$$
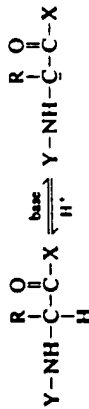
The same can be said about base-catalyzed racemization of amino acids. A negative charge on the carboxylate hinders further proton abstraction from the α-carbon of amino acids; dianions

$$\text{H}_2\text{N}-\overset{\text{R}}{\underset{\text{H}}{\text{C}}}-\overset{\text{O}}{\text{C}}-\text{O}^- + \text{OH}^- \xrightarrow{\;\;} \text{H}_2\text{N}-\overset{\text{R}}{\text{C}}-\overset{\text{O}}{\text{C}}-\text{O}^-$$

are not stable in protic solvents. Therefore, operations involving the amino acids themselves, for instance introduction of the benzyloxycarbonyl group, are carried out in distinctly alkaline solution. In fact, excess alkali prevents the formation of reactive derivatives, such as mixed anhydrides, which might be prone to racemization. In the absence of a free carboxyl group, as in alkyl esters of peptides, base catalyzed racemization does indeed occur during saponification with alkali.
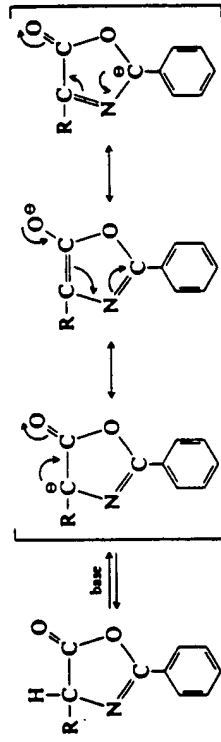
As indicated in the last paragraph, the *activated* carboxyl group poses the main problem in the preparation of optically homogeneous peptides. The electron-withdrawing effect of the activating group (x) extends to the α-carbon atom, the chiral center, and facilitates the abstraction of the hydrogen atom in the form of a proton

$$\text{Y}-\text{NH}-\overset{\text{R}}{\underset{\text{H}}{\text{C}}}-\overset{\text{O}}{\text{C}}-\text{X} \underset{\text{H}^+}{\overset{\text{base}}{\rightleftarrows}} \text{Y}-\text{NH}-\overset{\text{R}}{\text{C}}-\overset{\text{O}}{\text{C}}-\text{X}$$

This kind of simple proton abstraction is, however, not the sole, and not even the most common, mechanism of racemization. The most frequently invoked pathway involves cyclic intermediates, 4,5-dihydro-oxazole-5-ones or *azlactones*:
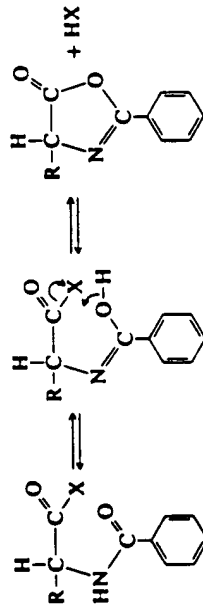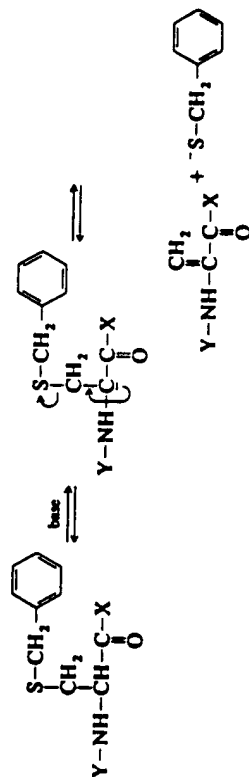

Proton abstraction from the chiral center yields a resonance stabilized anion

that was first postulated and subsequently proven by ir spectra. Azlactone formation is quite pronounced in benzoylamino acids, less prevalent in acetylamino acids and was for a long time thought to be absent in amino acids acylated by benzyloxycarbonyl or other urethane type blocking groups. The absence of racemization on activation of the latter was attributed to lack of azlactone formation, but in recent years azlactones were obtained from benzyloxycarbonyl-, tert.butyloxycarbonyl etc. amino acids as well. Furthermore, some optically pure azlactones were also prepared. Thus, azlactone formation itself is not a sufficient explanation of racemization; the stability of the cyclic intermediate toward bases must also be taken into consideration.

Activated derivatives of S-alkyl-cysteine suffer base catalyzed racemization even when their amino group is blocked by the benzyloxycarbonyl or other urethane-type protecting group. A simple, but not uncontested, explanation is reversible β-elimination
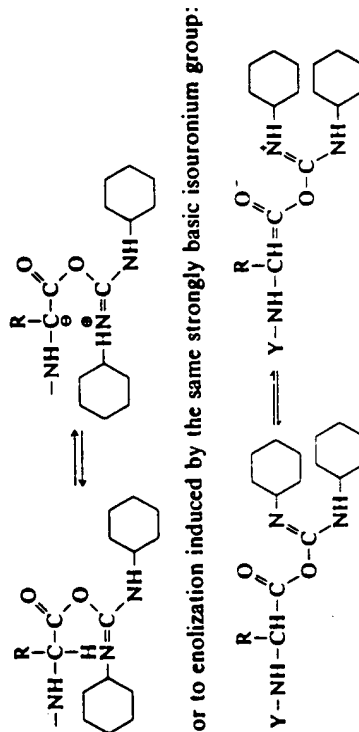

supported by the isolation of the thiobenzyl ester of N-benzyloxycarbonyl-S-benzyl-DL-cysteine. This indicates that benzylmercaptan, one of the products of β-elimination was present in the reaction mixture
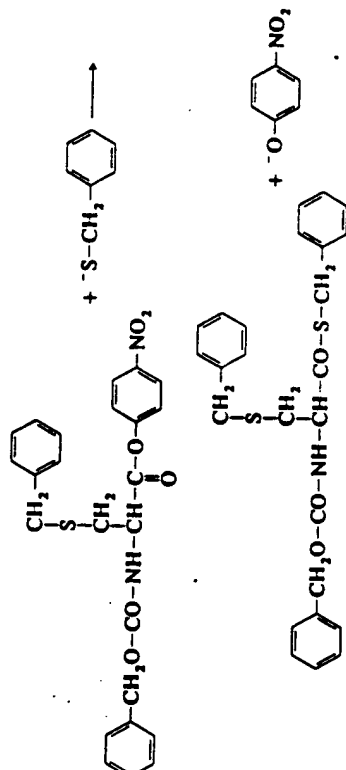
Experiments with S^{35} labeled benzylmercaptane, however, showed no incorporation of radioactivity. Also, racemization appears to be faster than deuterium exchange at the α-carbon atom. Thus racemization via β-elimination might occur only at elevated temperature, while other mechanism(s) could be opera-

tive under the conditions usually maintained during coupling. Among the various hypotheses that were put forward the partial acceptance of the negative charge of the carbanion intermediate by the d-orbitals of the sulfur atom is somewhat contradicted by the racemization of O-benzyl-serine derivatives.

The mechanisms described in the preceding paragraphs are the ones generally proposed for the explanation of racemization, but it is far from certain that other pathways are not involved. For instance it seems to be possible that the repeatedly observed loss of chiral integrity of the activated residue in coupling of peptides with the aid of dicyclohexylcarbodiimide is due to *intramolecular* proton abstraction by the basic center in the reactive O-acylisourea intermediate

or to enolization induced by the same strongly basic isouronium group:

Thus the acidic character of additives such as 1-hydroxybenzotriazole contributes to their ability to prevent racemization in coupling with carbodiimides.

B. Detection of Racemization

Loss of chiral homogeneity is an always present risk in peptide synthesis and there is an obvious need for methods that can reveal the presence of undesired diastereoisomers in the intermediates and particularly in the final product of the chain-building procedure. With carefully developed chromatographic systems it is often possible to separate fairly long peptide chains which are different from each other only with respect to the configuration of a single amino acid residue. There are however several methods available for this kind of scrutiny that can be applied without a special study of the particular product in question. Such general methods require hydrolysis of the peptide either with *acid* or with the aid of *proteolytic enzymes*. The specificity of these enzymes is the major advantage of the enzymatic approach: no hydrolysis occurs between a D-residue and the next amino acid in the sequence. Therefore complete hydrolysis will take place only in peptides that contain no D residues. The rate of peptide

bond fission, however, is a function of the amino acid cleaved from the N-terminus. Very slow rates can be achieved in the hydrolysis of glycyl and prolyl peptides with leucineaminopeptidase (a misnomer, since it is not specific for leucine), fewer difficulties are encountered with aminopeptidase M. Fast and complete hydrolysis of proline containing peptides requires the use of prolidases. A mixture of two enzymes, e.g. aminopeptidase M and prolidase can be quite efficient. Similar results are obtained with carboxypeptidases that provide stepwise removal of single amino acids starting with the C-terminal residue. Carboxypeptidase A has reduced catalytic effect when basic amino acids occupy the terminal position while carboxypeptidase B is most efficient just in this case. The yeast enzyme, carboxypeptidase Y is a more general catalyst.

A considerable number of biologically active peptides end with carboxamide rather than with a free carboxyl group. These peptide amides are, of course, no substrates for carboxypeptidases. An analogous problem exists in peptides which carry an acyl group such as the acetyl group at their N-terminus and, accordingly, can not be degraded with aminopeptidases. If both exopeptidase enzyme types fail, one can resort to a preliminary fragmentation of the chain with endopeptidases, such as trypsin. The latter is very specific for basic amino acids and catalyzes hydrolytic cleavage of the bond between arginine and the next residue and of the bond that follows lysine. The tryptic fragments then are suitable for further enzymatic degradation with exopeptidases, particularly with carboxypeptidase B. Chymotrypsin is similarly useful, but its specificity is somewhat less pronounced: in addition to the bond which follows an aromatic amino acid, the bond after a leucine residue is also cleaved, albeit at a slow rate. In hydrolyzates obtained with proteolytic enzymes only amino acids should be present; uncleaved peptides reveal the presence of a D-residue.
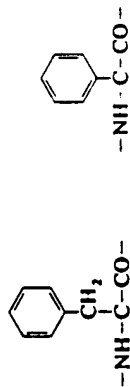
Acid catalyzed hydrolysis followed by the identification of D-amino acids in the hydrolysate is equally useful. To make this possible the amino acids in the mixture are acylated with an enantiomerically pure amino acid, for instance with the N-carboxyanhydride of L-leucine. In the resulting mixture of dipeptides any racemized residue is revealed by the formation of *two* dipeptides that are diastereoisomers of each other, for instance L-leucyl-L-phenylalanine and L-leucyl-D-phenylalanine. Since these are compounds with different physical properties they are separable and appear as a doublet on recordings of an amino acid analyzer. In recent years the conversion to diastereoisomers became unnecessary because the availability of chiral supports now permits separation of enantiomers by high pressure liquid chromatography (HPLC) and also by thin layer chromatography on plates covered with a chiral layer.

C. Racemization Studies in Model Systems

Racemization during the synthesis of peptides is a complex problem. The diversity of possible courses followed in the process is compounded by the
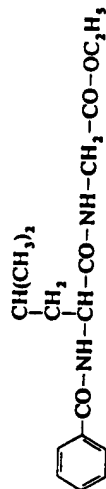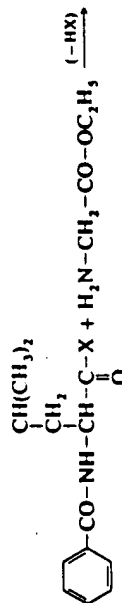
individuality of the amino acids. This was already shown in the example of S-alkyl-cysteine residues, which lose chiral purity by a special mechanism even if protected by a urethane-type protecting group that prevents racemization in other acylamino acids. The opposite end of the scale is represented by proline, which, at least under the commonly applied conditions of peptide synthesis, resists racemization. This was conventionally explained by the circumstance that proline is a secondary amine and, therefore, in its N-acyl derivatives lacks the hydrogen atom which participates in the formation of azlactones (cf. section A in this chapter). The experience, however, gained with readily racemized N-acyl derivatives of N-methylamino acids contradicts this assumption. It appears more likely that the rigidity of the cyclic side chain of proline excludes certain transition states that are integral parts of the racemization process.

Various side chains affect the extent of racemization in different ways. Thus, the benzyl side chain in phenylalanine contributes to the stabilization of a carbanion and can thereby facilitate proton abstraction from the α-carbon atom. This effect is much more pronounced in phenylglycine (which is not a protein constituent but occurs in microbial peptides) because its chiral carbon atom is benzylic:
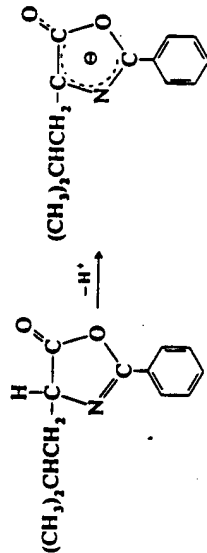
$$-NH-\overset{CH_2}{\underset{}{C}}-CO- \qquad -NH-\overset{}{\underset{}{C}}-CO-$$

The aliphatic side chains in alanine and leucine have no major influence but branching at the β-carbon atom in valine and isoleucine can enhance racemization because the combination of electron release and steric hindrance results in reduced coupling rates. The ensuing increase in the life-time of the reactive intermediate provides an extended opportunity for proton abstraction by base. It is obvious from these examples that the effect of individual side chains, the influence of various methods of coupling and the conditions of the peptide bond forming reaction (solvents, concentration, temperature, additives) must be studied in well designed experiments. Several model systems have been proposed for this purpose.

The first model (Williams and Young 1963) was based on coupling of benzoyl-L-leucine to glycine ethyl ester. The specific rotation of the crude product was used

$$Ph-CO-NH-\overset{CH_2-CH(CH_3)_2}{\underset{}{CH}}-\overset{}{\underset{O}{C}}-X + H_2N-CH_2-CO-OC_2H_5 \xrightarrow{(-HX)}$$

$$Ph-CO-NH-\overset{CH_2-CH(CH_3)_2}{\underset{}{CH}}-CO-NH-CH_2-CO-OC_2H_5$$

to establish enantiomeric purity. This simple system soon became popular and provided valuable information. Some shortcomings of the method must also be taken into consideration. The benzoyl group is not the best representative of blocking groups or of the part of the peptide chain that acylates the activated residue: it is more conducive to azlactone formation and might contribute to the stability of the anion generated from the azlactone by proton abstraction:

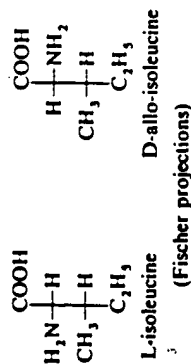$$(CH_3)_2CHCH_2-C\cdots \xrightarrow{-H^+} (CH_3)_2CHCH_2-C\cdots$$

Therefore the Young-test might lead to somewhat exaggerated estimates of racemization. This distortion is counterbalanced by the relative resistance of leucine to racemization, but an additional problem is created by the necessity of isolating the crude benzoyl-leucyl-glycine ethyl ester in excellent yield. If less than near quantitative yield is achieved in coupling or in recovery of the product, there remains the possibility that the unaccounted portion contains a not insignificant amount of the D-isomer.

A frequently used early model (Anderson and Callahan 1958) is based on the coupling of benzyloxycarbonyl-glycyl-L-phenylalanine to glycine ethyl ester. Since the phenylalanine residue is acylated by glycine and not by the benzyloxycarbonyl group, it is not protected against racemization. Accordingly, reactions which cause loss of chiral purity produce in addition to Z-Gly-L-Phe-Gly-OEt also its enantiomer Z-Gly-D-Phe-Gly-OEt. The extent of racemization is easily established from the amount of the racemate because it separates from dilute ethanol. However, the results of this test are reliable only if the peptide bond forming reaction proceeds with excellent yield. The presence of byproducts can grossly interfere with crystallization and no racemate might separate although the D-isomer has been produced in considerable amount. In general: it is risky to rely on negative evidence, the lack of separation of the racemate.

Several later model systems were designed with the thought of separating products that are not enantiomers but diastereoisomers of each other. For instance in the coupling of acetyl-L-isoleucine to glycine ethyl ester racemization will yield acetyl-D-alloisoleucyl-glycine ethyl ester, because inversion at the

α-carbon atom leads to a D-amino acid while chirality at the second chiral center, the β-carbon atom is unaffected

	COOH			COOH	
H_2N	—	H	H	—	NH_2
CH_3	—	H	CH_3	—	H
	C_2H_5			C_2H_5	

L-isoleucine   D-allo-isoleucine

(Fischer projections)

and hence, an alloisoleucine derivative is obtained. Complete hydrolysis (e.g. with 6 N HCl at 110° for 16 hrs) cleaves the amide and ester bonds and the hydrolysate can be applied to the column of an amino acid analyzer. In the well established Stein-Moore, method of amino acid analysis, isoleucine and alloisoleucine appear as well separated peaks and their ratio provides the information sought about the extent of racemization. The method does not require separation of the two peptides and therefore the results are not modified by imperfections in the operations of recovery. In more general versions of the same idea, diastereomeric tripeptides are produced, deblocked and compared with the help of the amino acid analyzer as such, that is without hydrolysis. For instance benzyloxycarbonyl-glycyl-L-alanine is coupled to L-leucine benzyl ester and after hydrogenation the mixture containing glycyl-L-alanyl-L-leucine and glycyl-D-alanyl-L-leucine is applied to the column of the instrument. By replacing L-alanine with other L-amino acids important information can be gained about the sensitivity of various amino acids to a certain coupling method or the conditions of coupling.

Volatile peptide derivatives, for instance trifluoroacetyl-L-valyl-L-valine methyl ester or benzyloxycarbonyl-L-leucyl-L-phenylalanyl-L-valine tert.butyl ester can be separated from their diastereoisomers that contain a D-residue by vapor phase chromatography. Also, through the examination of nmr spectra of relatively simple peptides the extent of racemization that occured during their preparation can be determined without separation of the diastereoisomers, because the difference in the chemical shifts of some selected resonances is sufficient for integration. Thus the areas under the well separated peaks of the alanine methyl protons in acetyl-L-phenylalanyl-L-alanine methyl ester and in acetyl-D-phenylalanyl-L-alanine methyl ester can be integrated and the values used to determine the extent of racemization of the phenylalanine residue during coupling.

These are only selected examples of the numerous model systems proposed for the study of racemization, yet, even in such a brief treatment an approach based on enantio-selective enzymes should be mentioned. Coupling of benzyl-oxycarbonyl-L-alanyl-D-alanine to L-alanyl-L-alanine benzyl ester yields a blocked intermediate from which on catalytic hydrogenation the free L-Ala-D-Ala-L-Ala-L-Ala is obtained. This compound is completely resistant to hydro-

lysis catalyzed by aminopeptidases, because the first bond to be cleaved links the N-terminal residue to a D-amino acid. If however, racemization took place during coupling, this changed the activated residue, D-alanine to L-alanine and after deblocking the tetrapeptide L-Ala-L-Ala-L-Ala-L-Ala is obtained. The latter is completely digestible with aminopeptidases. The liberated alanine is determined and it is a rather exact measure of racemization because for each residue inverted four molecules of alanine are found in the analysis.

At this point a comment has to be added concerning the degree of racemization established with the help of model systems. Usually the amount of the undesired diastereoisomer is considered to represent the extent of racemization. While this might be acceptable for the purpose of comparisons, one has to keep in mind that from the achiral intermediate of the process the two isomers formed in equal amounts. Thus the number of molecules involved is twice the number of the undesired diastereoisomers formed.
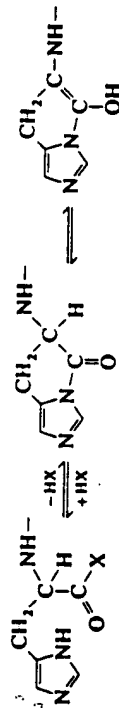
D. Prevention of Racemization

Since racemization during coupling is a base catalyzed process it is reasonable to assume that the nature of the base is not without influence on its outcome. Steric hindrance in some tertiary amines can weaken their ability to approach the chiral center in reactive intermediates. Diisopropylethylamine caused less racemization than triethylamine in the coupling of S-benzyl-L-cysteine derivatives, but it was without significant beneficial effect in reactions involving other amino acids. Perhaps in azlactones, the commonly implicated intermediates of racemization, the chiral carbon atom is well exposed and therefore the bulky groups in the tertiary amine can not interfere with proton abstraction. Also, some differences were found in couplings via mixed anhydrides between the previously preferred base triethylamine and tertiary amines such as N-ethylpiperidine or N-ethylmorpholine, the latter being less conducive to racemization. The principal lesson to be learned is, however, to omit, when possible tertiary amines from the coupling mixture. The often applied approach, addition of a tertiary amine to a salt of the amine component, is certainly inferior to the use of the amine component as such, that is the free amine. Several studies demonstrated that very little if any racemization takes place if this simple principle is followed.
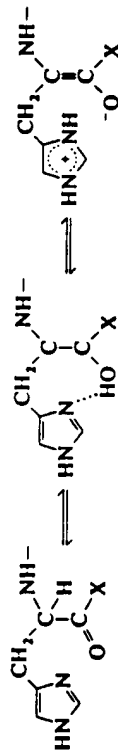
Tertiary amines are added to the reaction mixture also when mixed anhydrides are generated:

$$R\text{-}COOH + NR_3' \longrightarrow R\text{-}COO^- \cdot H\overset{+}{N}R_3'$$

$$R\text{-}COO^- \cdot H\overset{+}{N}R_3 + R''\text{-}\overset{\text{O}}{\overset{\|}{C}}\text{-}Cl \longrightarrow R\text{-}\overset{\text{O}}{\overset{\|}{C}}\text{-}O\text{-}\overset{\text{O}}{\overset{\|}{C}}\text{-}R'' + R_3'\overset{+}{N}H \cdot Cl^-$$

Understandably, production of the same mixed anhydrides is accompanied by less racemization if it is carried out with the help of 1-ethyloxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), because no addition of tertiary base is required and the basicity of the quinoline formed in the reaction is negligible. It is more difficult to counteract the effect of an intramolecular basic center, even if weak, such as the imidazole nucleus in the histidine side chain. While the here shown cyclization and enolization

do not eliminate the ability of the activated species to acylate the amine component (acylimidazoles are good acylating agents) the chiral integrity of the histidine residue may certainly suffer in the process. Racemization via enolization might occur without cyclization as well, particularly because the enol can be stabilized in enolate form:
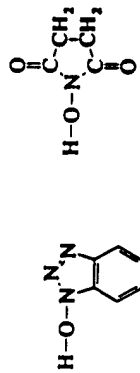
Hence it appears advantageous to further reduce the basic character of the imidazole by blocking, preferably at the π-nitrogen atom.
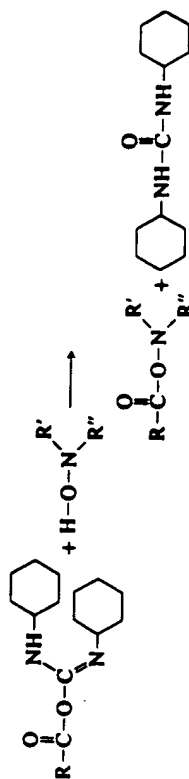
It is not surprising that a process involving proton abstraction is influenced by the polarity of the solvent. Base catalyzed racemization of active esters is fast in polar solvents such as dimethylformamide and slow in non-polar media, for instance in toluene. It is rather unfortunate that such non-polar solvents are more often than not impractical in peptide synthesis. The poor solubility of most blocked intermediates in the commonly used organic solvents severely limits their use and in the preparation of larger peptides indeed dimethylformamide is most frequently applied. The problem of solubility is less serious in solid phase peptide synthesis (cf. Chapter X), where no real solvent is needed but merely a medium in which the polymeric support properly swells. This function is fulfilled by dichloromethane; its effect on racemization lies between the extremes mentioned.

Proton abstraction from the chiral carbon atom can be suppressed by the addition of weakly acidic materials to the reaction mixture. Of the numerous additives tested 1-hydroxybenzotriazole (König and Geiger 1970a) and N-hydroxysuccinimide (Weygand et al. 1966) are routinely used in the practical execution of coupling. These compounds are not acidic enough to protonate the
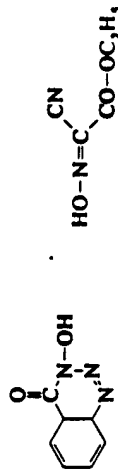
amino group

of the amine component and therefore they do not interfere with its acylation, but their acidity is sufficient to provide competition in abstraction of the proton from the carbon atom of activated intermediates. The significance of these additives is based however, not merely on their acidic character: many other weak acids perform poorly in the role of racemization suppressing agents. Both additives are related to hydroxylamine and function as powerful auxiliary nucleophiles. They react with overactivated intermediates, such as the O-acylisourea in carbodiimide mediated couplings,

reducing thereby the lifetime of the racemization prone species. The active esters produced in these reactions have higher chiral stability. In their reaction with the amine-component the additive is regenerated and assures a continued beneficial effect. Strangely, the highly efficient additives 3-hydroxy-3,4-dihydro-1,2,3-benzotriazine-4-one (König and Geiger 1970b) and 2-hydroximinocyanoacetic acid ethyl ester (Itoh 1973) have not been widely used so far although their effect on the prevention of racemization exceeds that of the popular 1-hydroxybenzotriazole.

The rather general measure that can be taken against side reactions, the use of both the carboxyl-component and the amine-component in high concentration, is applicable for the suppression of racemization as well. However, poor solubility of intermediates, sometimes even in dimethylformamide, presents a formidable obstacle compounded by the high molecular weight of some blocked peptides. Thus a high molar concentration of both components is often

unattainable. On the other hand, if the carboxyl component is not a peptide derivative but rather the blocked and activated form of a single amino acid, it can be used in excess. This excess can be adjusted to provide for a concentration which remains sufficiently high (for instance more than 0.1 molar) throughout the coupling reaction. The relative simplicity of the blocked and activated amino acid derivatives and their availability from commercial sources render this sacrifice usually acceptable. Over and above the possibility of performing coupling reactions according to the *principle of excess*, addition of single amino acid residues has a further important benefit in the conservation of chiral purity: the most commonly used amine-protecting groups, the benzyloxycarbonyl and the tert.butyloxycarbonyl group, efficiently prevent racemization in most cases. As mentioned before, this is a common feature of urethane-type blocking groups and applies for the acid-stable, base-sensitive 9-fluorenylmethyloxycarbonyl (Fmoc) group as well.

In the preceding discussion we have dealt only with racemization during peptide bond formation. Loss of chiral purity can occur, however, also during certain processes of deprotection. Hydrogenolysis is quite innocuous in this respect and acidolysis is harmful only if it is carried out under drastic conditions, such as elevated temperature. Saponification of esters with alkali can cause measurable racemization. This must be kept at a minimum by carrying out the reaction at ice-bath temperature, preferably at constant p_H. Large excess of alkali certainly must be avoided. The presence of Cu^{++} ions prevents racemization in alkaline hydrolysis (and probably also in many instances of coupling), but complete removal of the metal ions from the complex is not always straightforward.

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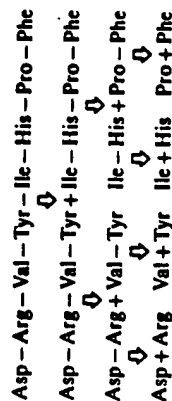
IX. Design of Schemes for Peptide Synthesis

In the strategical planning that must precede the synthesis of a larger peptide, racemization is one of the most important considerations. Therefore, it seems to be appropriate to discuss the various schemes of synthesis at this point. Due to the individuality of amino acid residues and to variations in the properties of blocked intermediates, it appears impractical to propose a general scheme (strategy) that would be applicable for any peptide. Peptide synthesis should be based on retrosynthetic analysis, starting with identification of the problems inherent in the sequence of the target compound.

In principle three approaches are possible: A. condensation of peptide segments; B. stepwise chain-building starting with the N-terminal residue; and C. stepwise chain building starting at the C-terminus. We will attempt to evaluate these alternatives, but with some reservation: there is no consensus among peptide chemists in this area.

A. Segment Condensation

In the earliest days of practical peptide synthesis, in the preparation of the nonapeptide oxytocin or the octapeptide angiotensin, segment condensation appeared to be the obvious strategy. Reduction of a major task to smaller problems, a Cartesian approach, is clearly attractive. Equally important is, however, the possibility of dividing the effort among members of a team. Preparation of the individual segments, often dipeptides, could be entrusted to less experienced coworkers while the arduous task of segment condensation needs an adept in peptide chemistry. A similar distribution of responsibilities is not feasible in stepwise chain lengthening. These considerations must have guided the investigators who undertook the synthesis of biologically active peptides in the nineteen fifties. The retrosynthetic scheme for the synthesis of the octapeptide angiotensin is shown here as an example:

Asp—Arg—Val—Tyr—Ile—His—Pro—Phe

Asp—Arg—Val—Tyr + Ile—His—Pro—Phe

Asp—Arg + Val—Tyr   Ile—His + Pro—Phe

Asp + Arg   Val + Tyr   Ile + His   Pro + Phe

Miklos Bodanszky

Principles
of Peptide Synthesis

Second, Revised Edition

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Preface to the Second Edition

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Princeton, NJ 08540, USA

The attempt to render PRINCIPLES OF PEPTIDE SYNTHESIS somewhat resistant to the passing of time could, of course, be only partially successful. In the decade that has elapsed since the completion of the manuscript, the discovery of a long series of biologically active peptides together with the major application of peptide hormones, such as calcitonin, the blood-pressure-lowering enzyme inhibitor, the pseudopeptide captopril, in medicine, and the large-scale production of the sweetener, aspartame, have given new impetus to peptide chemistry. A considerably widening of interest in peptide synthesis, both in academia and in industry, ensued and numerous novel methods appeared in the literature. It seemed timely to update the original version of PRINCIPLES OF PEPTIDE SYNTHESIS.

Preparation of this Second Edition provided a welcome opportunity for revising the text. This revision went beyond the correction of printer's errors and other mistakes. A more substantial modification of the first edition was prompted by a thorough critique by Professor G.T. Young of Oxford University. I considered his recommendations carefully and adopted most of them. Some changes in the evaluation of methods have also been made. For instance I reexamined the principle of coupling reagents and introduced the concept of "true coupling reagent".

Only a part of the new procedures could be fitted into the appropriate chapters of the first edition, hence most of the material published between 1982 and 1992 was assembled in the concluding Chapter VIII. This separation of old and new served not merely convenience but also allowed me to attempt an assessment of new ideas and to discern novel trends.

Princeton, New Jersey, 1993   MIKLOS BODANSZKY

In esters and in various activated derivatives of acylamino acids, no such obstacle exists against proton abstraction. In fact the electron withdrawing forces present in the activating group "X" enhance the activity of the α-hydrogen and facilitate its abstraction:

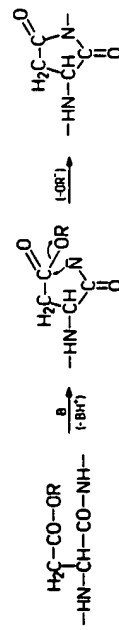$$-\text{HN}-\underset{\text{H}}{\overset{\text{R}}{\text{C}}}-\text{CO}-\text{X} \underset{(-\text{BH}^+)}{\overset{\text{B}}{\rightleftharpoons}} -\text{HN}-\overset{\text{R}}{\text{C}}-\text{CO}-\text{X}$$

An obvious consequence of carbanion formation is the partial or total loss of chiral purity. Proton abstraction might be reversible and the equilibrium of the reaction might lie far to the left: gradually more and more molecules will pass through a carbanion stage and suffer irreparable racemization. Therefore, the risk of racemization is inherent in peptide synthesis and in order to avoid it, it must be carefully considered. There are, however, side reactions in which proton abstraction occurs not at the α-carbon atom but at the amide nitrogen of an acylamino acid. The additional

$$\overset{\text{O}}{\underset{\text{H}}{\overset{\|}{\text{C}}}}-\overset{}{\text{N}}-\text{CHR}-\text{CO}- \underset{(-\text{BH}^+)}{\overset{\text{B}}{\rightleftharpoons}} -\overset{\text{O}}{\overset{\|}{\text{C}}}-\overset{}{\text{N}}-\text{CHR}-\text{CO}-$$

unshared pair of electrons on the nitrogen atom renders the latter, in spite of the presence of the carbonyl substituent, a good nucleophile. Thus, it can participate in numerous side reactions, particularly in intramolecular attacks resulting in cyclizations. For instance, the formation of succinimide derivatives is usually preceded by proton abstraction from the amide nitrogen of an aspartyl amino acid residue:

$$\begin{array}{c}\text{H}_2\text{C}-\text{CO}-\text{OR}\\ -\text{HN}-\text{CH}-\text{CO}-\text{NH}-\end{array} \underset{(-\text{BH}^+)}{\overset{\text{B}}{\rightleftharpoons}} \begin{array}{c}\text{H}_2\text{C}-\text{C}\overset{O}{\big\langle}_{\text{OR}}\\ -\text{HN}-\text{CH}\overset{}{\big\langle}_{\overset{}{\text{O}}}^{\overset{}{\text{N}}}\end{array} \overset{(-\text{OR}^-)}{\longrightarrow} \begin{array}{c}\text{H}_2\text{C}-\text{C}\overset{O}{\big\langle}\\ -\text{HN}-\text{CH}\overset{}{\big\langle}_{\overset{}{\text{O}}}^{\overset{}{\text{N}}-}\end{array}$$

Analogous cyclization reactions and O-acylations initiated by proton abstraction will be discussed in separate sections.
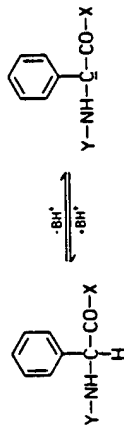
1.1   Racemization

1.1.1   Mechanisms of Racemization

Understanding the mechanisms of racemization seems to be necessary for its prevention. Accordingly, a considerable amount of experimental work has been carried out in this area, and was skillfully rendered in a review article by Kemp [4]. At this place we confine the discussion to the

principal processes of base catalyzed racemization of activated acylamino acids. Three distinct pathways can be recognized:
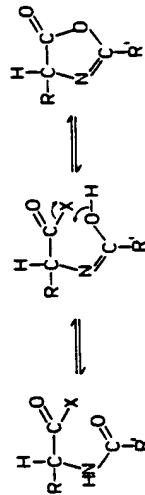
a) direct abstraction of the α-proton,
b) racemization via reversible β-elimination and
c) racemization through azlactones [5(4H)-oxazolones].

The simple proton abstraction mechanism might be a contributor in several processes but it is the dominant pathway only in very special cases such as the rapid racemization of derivatives of phenylglycine, an amino acid which is not a constituent of proteins although it occurs in microbial peptides:
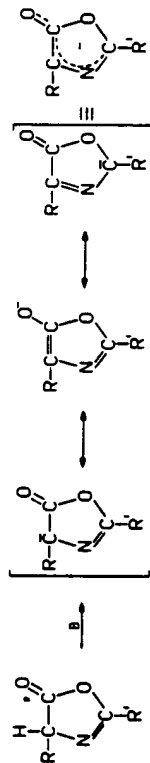
$$\text{Y}-\text{NH}-\underset{\text{H}}{\overset{\text{C}_6\text{H}_5}{\text{C}}}-\text{CO}-\text{X} \underset{-\text{BH}^+}{\overset{+\text{BH}^+}{\rightleftharpoons}} \text{Y}-\text{NH}-\overset{\text{C}_6\text{H}_5}{\text{C}}-\text{CO}-\text{X}$$

(where Y is a protecting group and X an activating group). The conspicuous racemization of active esters of N-benzyloxycarbonyl-S-benzyl-L-cysteine [5] was usually explained by the reversible, base-induced elimination of benzylmercaptane and this explanation was supported by the fortuitous isolation of N-benzyloxycarbonyl-S-benzyl-DL-cysteine thiobenzylester from a solution of the p-nitrophenyl ester containing triethylamine. Subsequent studies, carried out e.g. with radioactively labelled benzylmercaptane [6], demonstrated that racemization of reactive cysteine derivatives can proceed without the elimination of the thiol. Further examination of the problem led to a proposal [7] in which a direct interaction between the chiral carbon-atom and the sulfur atom, involving the d-orbitals of the latter, is invoked. The problem, however, is further complicated by the often observed racemization of reactive derivatives of O-benzyl-serine (in which clearly no d-orbitals are present) during coupling. Thus the d-orbitals of the sulfur atom might contribute to but cannot be solely responsible for the racemization of S-alkyl-cysteine. A more acceptable rationale, applicable both for cysteine and for serine derivatives, is enol-stabilization by intramolecular hydrogen bonds, with the sulfur or the oxygen atom, respectively, as bridgeheads. The ready racemization noted in reactive derivatives of β-cyano-alanine [8] can be explained simply by the strong electron-withdrawing effect of the cyano group.
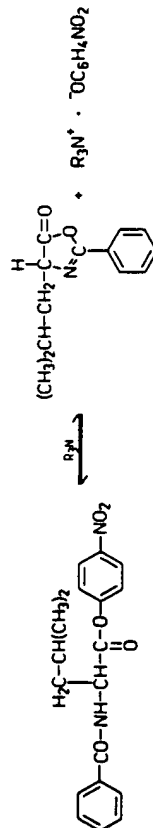
The best studied and probably most important mechanism of racemization involves the formation of azlactones [9]:

$$\begin{array}{c}\text{R}-\text{C}\overset{O}{\big\langle}\\ \text{R}'-\text{C}-\text{N}-\text{CO}-\text{R}''\\ \text{H}\quad\text{H}\end{array} \rightleftharpoons \begin{array}{c}\text{R}-\text{C}\overset{O}{\big\langle}\\ \text{R}'-\overset{\text{H}}{\text{C}}-\text{N}=\text{C}\overset{\text{X}}{\big\langle}_{\text{R}''}\end{array} \overset{-\text{O}-\text{H}}{\rightleftharpoons}$$

$$\begin{array}{c}\text{R}-\overset{\text{H}}{\text{C}}-\text{C}\overset{O}{\big\langle}\\ \text{R}'\quad\text{HN}-\text{C}\overset{}{\big\langle}_{\text{R}''}^{\text{O}}\end{array} \rightleftharpoons \begin{array}{c}\text{R}-\overset{\text{H}}{\text{C}}\overset{}{\big\langle}\\ \text{R}''-\text{C}\overset{}{\big\langle}_{\text{O}}^{\text{N}}\end{array}$$
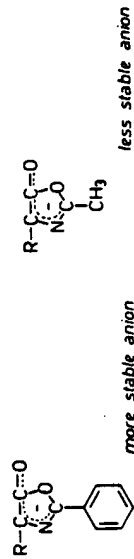
The explanation for the tendency to racemization of azlactones lies in the ease by which the acidic proton can be abstracted by bases from the chiral center due to resonance stabilization of the carbanion generated in the process:

Azlactones are good acylating agents and could be useful for the activation of the carboxyl component. Yet, delocalization of the negative charge in the deprotonated intermediate provides them with sufficient lifetime to endanger the chiral purity of the product. The formation of an azlactone could be demonstrated [10] by its characteristic carbonyl frequency (1832 cm^{-1}) when benzoyl-L-leucine p-nitrophenyl ester was exposed to the action of tertiary amines
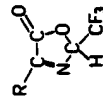
and equally convincing evidence incriminating the azlactone intermediate was found in the production of partially racemized benzoyl-leucyl-glycine ethyl ester when the reaction was completed with acylation of glycine ethyl ester. Characteristically, the unreacted portion of benzoyl-L-leucine p-nitrophenyl ester was recovered enantiomerically pure. Racemization through azlactone intermediates is influenced by several factors such as the nature of the amino acid involved, the solvent used in the reaction or the presence (or absence) of tertiary amines. The acyl group on the amine nitrogen, however, plays a decisive role in the conservation or loss of chiral purity. For instance, under identical conditions, benzoylamino acids are more extensively racemized than acetylamino acids [11]. Such differences seem to be related to the electronic forces operating in the acyl group. Beyond the formation of azlactones the N-acyl substituents of the oxazolinone can also affect the acidity of the hydrogen atom on the chiral center. Expressed in another way: the stability of the anion produced in proton abstraction by bases is enhanced by electron withdrawing effects in the acyl group:
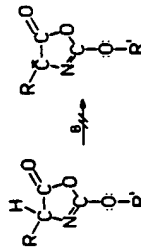
more stable anion   *less stable anion*

It is probably not so much the formation of azlactones that is of primary importance in determining the rate of racemization but rather the electronic effects of the substituents of the oxazolinone, including those in the N-acyl group. Azlactones can be obtained in optically active form [12], and if immediately trapped by good nucleophiles [13], they can yield optically active products.

The influence of the N-acyl group on the stability of the anion generated through proton abstraction from the oxazolinone can range from extreme stabilization found in the formyl and trifluoroacetyl groups to pronounced destabilization shown by the benzyloxycarbonyl, tert-butyloxycarbonyl and other alkoxycarbonyl groups. In fact, trifluoroacetylamino acids yield an isomer [14] of the more common azlactones, an isomer in which the α-carbon atom is not chiral:
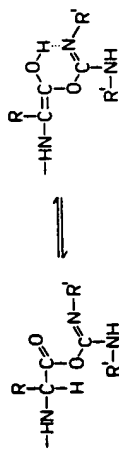
Until recently it was generally assumed that benzyloxycarbonylamino acids and, in general, amino acids protected by a urethane-type blocking group do not produce azlactones and hence are resistant to racemization during activation and coupling. Isolation [15] of optically pure oxazol-(4H)-ones, e.g. from the reaction of tert-butyloxycarbonyl-L-valine with water soluble carbodiimides contradicts such assumptions and suggests that the beneficial effect of urethane type protecting groups rests on the electron release provided by them and on the ensuing destabilization of the anion which could form by proton abstraction:
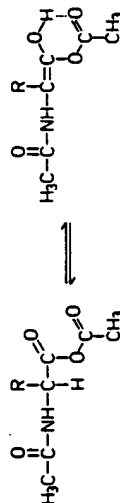
The chiral stability of proline derivatives was usually explained by the absence of an amide hydrogen in the N-acyl derivatives of this secondary amine. It appeared plausible that without such an amide hydrogen no azlactone should form. This explanation, however, ignores the possible

formation of protonated azlactones (oxazolonium salts). It was completely refuted by the ready racemization of N-methylamino acids [16] during activation and coupling. Thus, the chiral stability of proline is due to its rigid geometry rather than the fact that it is a secondary amine. Under certain conditions, e.g. in diketopiperazines, proline is readily racemized.

The role of bases in at least some of the racemization processes is beyond doubt. For instance, time and again the advantage of free amines over a mixture of amine salts with tertiary bases was noted. Less attention has been paid so far to the possibility of *intramolecular* base catalysis, although in several coupling methods the reactive intermediate contains a basic center and the latter could abstract the hydrogen from the chiral carbon atom. Since O-alkyl isoureas have pronounced basic character, it may not be farfetched to assume intramolecular proton abstraction by a basic nitrogen atom in the O-acyl-isourea intermediates of carbodiimide mediated coupling reactions. For instance hydrogen bond stabilized enols might play a role in such processes

$$-HN-\underset{R}{\overset{}{C}}-C=O \qquad \longrightarrow \qquad -HN-C=C-O-H$$

which would then be analogous to the effect of excess acetic anhydride on optically active amino acids. Here racemization probably proceeds through enolization of mixed anhydrides:

$$H_3C-C-NH-\underset{R}{\overset{O}{C}}-C=O \qquad \rightleftharpoons \qquad H_3C-C-NH-C=C-O-H$$
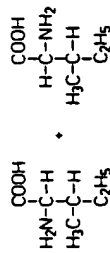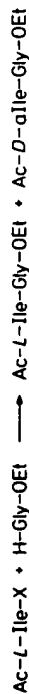
1.1.2   Models for the Study of Racemization

Numerous model systems have been proposed for the study of racemization. These systems are used to evaluate the effect of solvents, presence or absence of bases, temperature and other variables and last, but not least, the ability of different coupling methods to produce peptides without loss of chiral purity. The earliest suggestions came from Young's laboratory [17, 18] and involve the coupling of acetyl or benzoyl-L-leucine to glycine ethyl ester, followed by the examination of the optical rotation of the crude product. The results can be further refined by fractional crystallization

and analysis of the fractions by weight, optical rotation and melting point. The benzoyl group enhances the tendency for racemization, hence activation and coupling of benzoyl-L-leucine is a very sensitive racemization test.

A simple, and therefore frequently applied, model experiment was designed by Anderson and Callahan [19]. It involves the coupling of benzyloxycarbonylglycyl-L-phenylalanine to glycine ethyl ester. If racemization occurs in the process the product contains benzyloxy-carbonylglycyl-DL-phenylalanyl-glycine ethyl ester, which is fairly insoluble in aqueous ethanol and can thus be separated and weighed. A word of caution is indicated here. This simple and useful method is reliable only if no by-products, other than the racemate, are formed in significant amount in the coupling reaction. Otherwise crystallization of the racemate might be impeded by the impurities and from the lack of crystallization the wrong conclusion, that there was no racemization, can be drawn. In principle, models should be so designed that the products of the test-experiment are not racemates but diastereoisomers and the conclusions are not based on negative evidence.

A more reliable, albeit also more time consuming, experiment is based on the coupling of benzyloxycarbonylglycyl-L-alanine to L-phenylalanyl-glycine ethyl ester (the "Kenner model") [20]. The diasteroisomers formed in the reaction are separated by countercurrent distribution. Somewhat less laborious are the methods introduced by Weygand and his associates [14, 21, 22], who condensed trifluoroacetyl-L-valine with L-valine methyl ester, or benzyloxycarbonyl-L-leucyl-L-phenylalanine with L-valine *tert*-butyl ester or trifluoroacetyl-L-prolyl-L-valine with L-proline methyl ester. The reaction products are examined with the help of vapor phase chromatography for the presence of diastereoisomers formed by racemization.
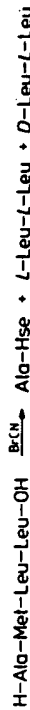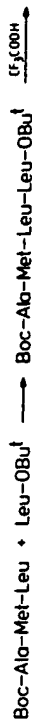
The test systems discussed so far are based on differences with respect to solubility or partition coefficient between diastereoisomers (or in the Anderson-Callahan test, between the racemate and the enantiomerically pure peptide derivative). An experimentally simple realization of the same principle is the examination of the products of model reactions by paper chromatography or thin layer chromatography [23]. Improvements in the realiability of the tests are also possible, e.g. the Young test can be perfected by the chromatographic separation of the products [24]. A more substantial simplification is, however, the use of the ubiquitous amino acid analyzer for the separation and quantative determination of the diasterosimers generated in the racemization tests. For instance coupling of acetyl-L-isoleucine [25] to glycine ethyl ester yields, in addition to the desired acetyl-L-isoleucylglycine ester, also acetyl-D-*allo*isoleucylglycine ethyl ester, if racemization occured in the reaction. Since alloisoleucine and isoleucine are routinely separated by the Spackman-Stein-Moore method [26], it is sufficient to

Ac-L-Ile-X + H-Gly-OEt ⟶ Ac-L-Ile-Gly-OEt + Ac-D-alle-Gly-OEt

$$\xrightarrow{H^+/HOH} \quad H_2N\text{-}CH_2COOH \;+\; \begin{array}{c}COOH\\H_2N\text{-}C\text{-}H\\H_3C\text{-}C\text{-}H\\C_2H_5\end{array} \;+\; \begin{array}{c}COOH\\H\text{-}C\text{-}NH_2\\H_3C\text{-}C\text{-}H\\C_2H_5\end{array}$$

hydrolyze a small sample of the reaction mixture and to apply the hydrolysate to the analyzer. The main advantage of this model experiment is that no isolation of products is needed. This means a certain saving of time and effort, but more importantly the examination of the *crude* material assures that no distortion takes place in the isolation or separation of the products, thus no isomer is left in mother liquors, etc. The acetyl group has no major effect on the racemization of the amino acid to which it is attached, thus in this respect it can represent a peptide chain. This model can be applied for the study of the effect of coupling methods, solvents, tertiary amines added and also of the influence of the amino component, since glycine ethyl ester can be replaced by other nucleophiles. Yet, a certain limitation is caused, by the choice of isoleucine as the activated residue. It is a hindered amino acid and might suffer more loss in chiral purity than other less hindered residues which do not reduce the rate of the desired reaction and therefore allow less time for unimolecular processes such as racemization.

The same principle, separation of diastereoisomers on the amino acid analyzer, appears also in the "Izumiya test" [27, 28] in which a benzyloxy-carbonylglycyl amino acid is coupled to an optically active amino acid benzyl ester and the products examined after deprotection by hydrogenation. This model system allows variations with respect to the amino acid residue which is exposed to racemizing conditions. Thus, instead of Z-Gly-L-Ala one can couple Z-Gly-L-Phe, etc. to L-Leu-OBzl and the nucleophile can also be so selected that detection of the diastereoisomers causes no difficulty. The contributions of Benoiton and his associates [29, 30], who used N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester for amino component, lie in the same direction. The degree of racemization can be estimated, without deprotection and separation, through the examination of the nmr spectra of the coupling products. The model compounds acetyl-L-alanyl-L-phenylalanine methyl ester and acetyl-L-phenylalanyl-L-alanine methyl ester [31] allow the determination of the D-amino acid containing isomers by integration of the areas of the methyl protons of alanine while coupling of benzoylamino acids to N^{ε}-benzyloxycarbonyl-L-lysine methyl ester [32] permits a similar assessment of racemization through the examination of the methyl protons of the methyl ester group. In an interesting proposal [33] coupling of *tert*-butyloxycarbonyl-L-alanyl-L-methionyl-L-leucine to the *tert* butyl ester of L-leucine is followed by acidolysis and then by a treatment with cyanogen bromide in aqueous

acetic acid and by determination of the ratio of the two diastereoisomers, L-Leu-L-Leu and D-Leu-L-Leu with the help of the amino acid analyzer:

Boc-Ala-Met-Leu + Leu-OBu^t ⟶ Boc-Ala-Met-Leu-Leu-OBu^t $\xrightarrow{CF_3COOH}$

H-Ala-Met-Leu-Leu-OH $\xrightarrow{BrCN}$ Ala-Hse + L-Leu-L-Leu + D-Leu-L-Leu

Hse = Homoserine

In a sophisticated and also very sensitive model experiment [34] benzyloxycarbonyl-L-alanyl-D-alanine is activated by the method to be tested and coupled to L-alanyl-L-alanine p-nitrobenzyl ester. The crude product is deblocked by hydrogenation and the mixture of the two isomeric tetrapeptides L-Ala-D-Ala-L-Ala-L-Ala and L-Ala-L-Ala-L-Ala-L-Ala, is exposed to the action of leucine aminopepidase. The enzyme will catalyze the complete hydrolysis of the all-L peptide, the product of racemization, but leaves the peptide in which the second position is occupied by a residue with D-configuration intact. With respect to sensitivity this method is surpassed by the isotope dilution techniques introduced into peptide chemistry by Kemp and his coworkers [35–37]. Radioactively labeled benzyloxycarbonylglycyl-L-leucine or benzoyl-L-leucine is coupled to glycine ethyl ester followed by dilution with "cold" racemate and fractional crystallization until products with constant count per mg are obtained. This yields reliable information on racemization and allows the detection of very slight racemization which would be left unnoticed in the original versions of the Anderson-Callahan or the Young tests (cf. above).

Some problems, e.g. the base catalyzed racemization of active esters of protected amino acids or peptides can be investigated simply by following the change of optical rotation with time [38]. The effect of solvents, protecting groups, temperature, activating groups, etc. can be studied in this simple manner. With selected model compounds [39] it was possible to determine the scope and limitations of hindered amines in preventing racemization.

1.1.3   Detection of Racemization (Examination of Synthetic Peptides for the Presence of Unwanted Diastereoisomers)

Racemization during the activation and coupling of suitably protected amino acids occurs rarely but cannot be excluded. It is even more likely to occur in the activation and coupling of protected peptides. Therefore, it is desirable and sometimes absolutely necessary to examine the synthetic products for the presence of unwanted diastereoisomers. Such contaminants, if they are only minor constituents in the crude synthetic material, might be lost in the isolation process or during purification but can also accompany the principal product through these steps. A simple and

enzymes from snake venoms. The evidence obtained in these oxidative processes should be trusted only if the catalytic effect of the enzyme preparation and the conditions used are shown to be operative in control experiments with mixtures containing both L and D amino acids.

Chromatographic procedures based on columns containing chiral supports [46, 47] can differentiate between D and L amino acids. This principle, perfected by the use of high pressure liquid chromatography, might become the standard control process for the detection of racemization that occurred in the synthesis of a peptide. Reversed phase high pressure chromatography is well suited [48] also for the implementation of the Manning-Moore procedure [40] because well selected columns can completely separate the diastereoisomers formed on acylation of the amino acids in a hydrolysate with an optically pure acylating agent.

1.1.4 Conservation of Chiral Purity

Chiral purity of activated residues is affected by several factors, such as the methods of activation and protection or the nature of the activated amino acid residue. It is influenced also by the solvent used in the reaction, the presence or absence of tertiary amines, and by the basic strength and bulk of the tertiary amine if one had to be added to the coupling mixture and, last but not least, by auxiliary nucleophiles (cf. Chapter II). First and foremost of these factors seemed to be *the method of activation* and thus it received the most attention. The search for "racemization free" coupling methods is still actively pursued although this effort is fraught with an inherent difficulty. Any increase in the activation of the carboxyl group entails an increase in the acidity of the proton on the chiral α-carbon atom and facilitates, thereby, racemization via proton abstraction:

$$-HN-\underset{H}{\overset{R}{C}}-\overset{O}{\underset{}{C}}{\textstyle\diagdown}_X + B \; \rightleftharpoons \; -HN-\overset{R}{\underset{}{C}}-\overset{O}{\underset{}{C}}{\textstyle\diagdown}_X + BH^+$$

It might be more profitable to focus attention on each and every factor influencing racemization, rather than to try to develop perfect coupling methods which will yield chirally pure products under any conditions.

Through decades the strong belief prevailed that the azide method is free from racemization. Only later did we become aware of measurable racemization in azide coupling [21, 49, 50]. Those who observed no racemization in the preparation of peptides via azides (e.g. Ref. [27]) knowingly or intuitively avoided the use of tertiary bases, or at least did not apply tertiary amines in excess [51]. By no means do we suggest that all methods are equal in this respect. The azide method still stands out as

practical approach to the detection of diastereoisomers was devised by Manning and Moore [40]. A sample of the peptide is completely hydrolyzed with constant boiling hydrochloric acid and the mixture of liberated amino acids is acylated with an enantiomerically pure protected and activated amino acid, e.g. with L-leucine N-carboxy-anhydride. The resulting mixture of dipeptides is applied to the column of an automatic amino acid analyzer [26] which can separate dipeptides from their diastereoisomers. Accordingly, if racemization occurred at one or more residues, then, in addition to the peaks corresponding to the expected dipeptides (L-leucyl-L-amino acids) smaller satellite peaks will also appear on the recordings, demonstrating the presence of L-leucyl-D-amino acids in the mixture. The areas under the peaks allow the quantitative determination of the amount of D-amino acids in the synthetic material. There is, of course, an inherent limitation in the examination of chiral integrity of a peptide through its hydrolysis with acids, if the process of hydrolysis itself is not unequivocal in this respect. In acid hydrolysates, most amino acids appear more or less intact, but some, e.g. phenylalanine, suffer minor racemization during hydrolysis, while cystine becomes heavily contaminated with its D-isomer and also with mesocystine. Alkaline hydrolysis is even worse. It causes extensive racemization in several residues. Such details must be taken into consideration in the evaluation of the Manning-Moore analysis. This problem can be eliminated by using proteolytic enzymes for degradation.

The selectivity of proteolytic enzymes also permits their direct application for the study of optical homogeneity [41]. For instance complete digestibility of a sample with leucine amino peptidase [42, 43] provides strong evidence for the absence of D-amino acid containing peptides. A comparison of the ratios of amino acids in hydrolysates obtained on digestion of a synthetic product with proteolytic enzymes with the ratios determined in a routine acid hydrolysate is probably one of the simplest approaches for the study of chiral integrity.
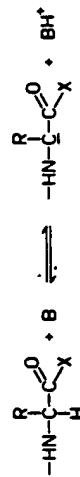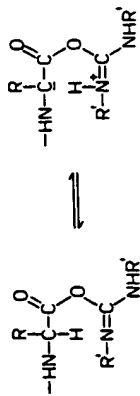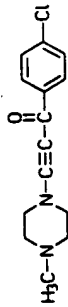
The rates of hydrolysis in degradation with proteolytic enzymes are usually low at bonds following proline and glycine residues. Some aminopeptidases, e.g. aminopeptidase M, are less restrictive in this respect. Proline, a stumbling block in proteolysis, can be set free with the help of specific prolidases [44, 45]. In addition to aminopeptidases, carboxypeptidases A, B and Y, and dipeptidylaminopeptidases can also be adopted for the same purpose. Selective cleavage, e.g. with trypsin at the carboxyl side of arginine and lysine residues, provides useful information if these were the activated amino acids of carboxyl components. In general, the stereospecificity of enzyme catalyzed hydrolysis can serve the study of optical purity in numerous ways. Perhaps less reliable is an alternative approach in which one follows the disappearance of D-amino acids from hydrolysates on treatment with D-aminoacid oxydases (e.g. from kidneys) or the elimination of L-amino acids by oxidation with

less conducive to racemization than many other procedures, but probably even the best methods can cause racemization under adverse conditions.
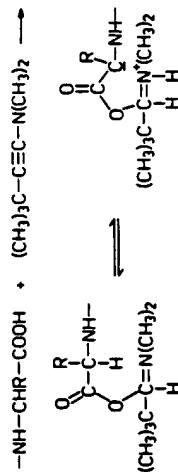
In the choice of coupling methods it is difficult to make positive recommendations, although some procedures, e.g. coupling via azides or with the help of EEDQ [52] have a fairly good record. It might be easier to point out coupling reagents which are notorious for their ability to cause racemization. Some of these, for instance the Woodward reagent [53], dicyclohexylcarbodiimide and other carbodiimides [54] caution the investigator by the structure of the reactive intermediates which contain a basic center, the potential cause of intramolecular proton abstraction from the chiral carbon atom:
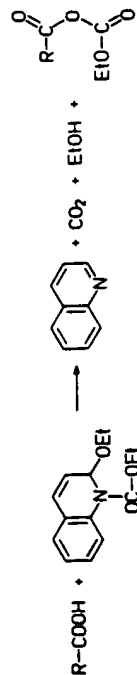
Similarly, among the various "push-pull acetylenes" [55–57] one with two basic centers [56]

is more conducive to racemization than others with only a single proton abstracting site. A basic center is generated in the earlier proposed [58] ynamines as well:
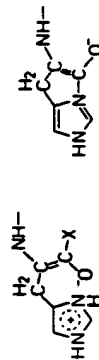
These considerations suggest that the lesser tendency of certain procedures to cause racemization is related to the absence of proton abstracting centers in the reactive intermediate and/or to the generation of materials which provide protons more readily than the chiral center of the activated residue. Thus, EEDQ [52] yields alcohol (and quinoline which has negligible basic strength):
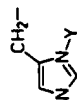
In several coupling methods substances are released which are not acidic enough to prevent acylation of the amino component, but which can, nevertheless, effectively compete with the chiral center as proton donors. This is the situation with active esters which liberate substituted phenols or hydroxylamines during coupling.

In the base catalyzed racemization of reactive intermediates the amount and concentration of the base play an obvious role. The general principle to avoid basic conditions is supported by numerous reports and hardly requires further evidence. Thus, a free amine as nucleophile is preferable to a mixture of a salt of the amino component with a tertiary base. Weak acids, e.g. 1-hydroxybenzotriazole, do not interfere with acylation and coupling can be carried out without the addition of a tertiary amine [3]. Yet, over and above the *amount of the organic base* added to the reaction mixture its *chemical character* also has significant influence on the outcome of acylation. For instance, in mixed anhydride reactions, *N*-methylmorpholine causes less racemization [49] than the widely used triethylamine. In coupling via azides 1-diethylamino-2-propanol was found to be harmless [51] while triethylamine, *N*-methylmorpholine and diisopropylethylamine had, under certain conditions, an unfavorable effect on chiral purity. The last mentioned base prevents [39] the racemization of active esters of benzyloxycarbonyl-L-phenylglycine and of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine, but had an almost as unfavorable effect on the optical purity of benzoyl-L-leucine *p*-nitrophenyl ester as other, less hindered, tertiary amines. Apparently steric hindrance in diisopropylethylamine is insufficient to interfere with proton abstraction from azlactone intermediates. Tribenzylamine seems to be more efficient in this respect. It is quite possible, however, that the influence of bases on racemization is determined not solely by their bulkiness but also by their basic strength [59, 60]. In this connection the racemization enhancing effect of the highly nucleophilic base *p*-dimethylaminopyridine [62, 63] should also be mentioned. On the other end of the scale, the weakly basic imidazole affects unfavorably the outcome of coupling reactions, particularly if its action is intramolecular. Thus, in acylation with activated derivatives of histidine significant racemization was observed [64], presumably caused by base catalyzed enolization or by cyclization and enolization.
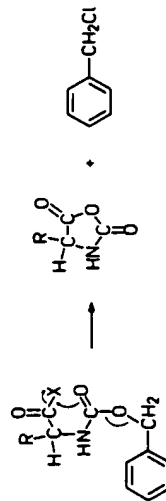
Substituents which reduce the basicity of the imidazole nucleus, e.g. the *p*-toluenesulfonyl group [65], reduce the extent of racemization as well [66]. Yet, a complete protection against loss of chiral purity of histidine residues can be expected only in derivatives in which the side chain protecting group (Y) is on the π-nitrogen atom of the imidazole:
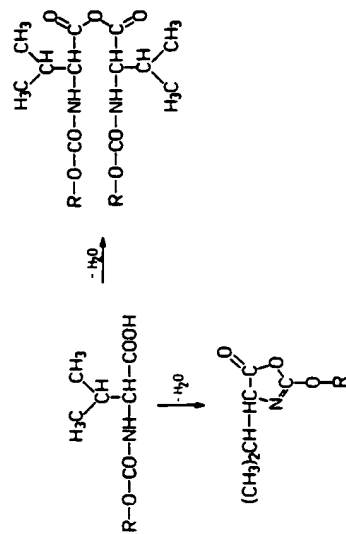
CH$_2$—

Among the factors which determine racemization the polarity of the *solvent* is quite important [4, 14]. In general, racemization is fast in highly polar solvents such as hexamethylphosphoramide, dimethylsulfoxide or dimethylformamide and is less pronounced in less polar solvents, e.g. pyridine, acetonitrile, chloroform, dichloromethane, tetrahydrofuran, dioxane or toluene. Unfortunately, most peptide intermediates are not sufficiently soluble in non-polar solvents and, at this time, the majority of acylation reactions are carried out in dimethylformamide. In solid phase peptide synthesis one applies solvents in which the peptidyl resin swells and a dissolution of the reactants is not needed. Thus, dichloromethane, which is not particularly conductive to racemization, can be used. An additional problem is created, however, by the solvent dependence of the rate of acylation of various activated intermediates. The most commonly used active esters react far better in polar solvents than in non-polar ones. These circumstances render the selection of solvents which would be favorable for acylation and yet cause little damage to chiral purity, rather difficult. A general remedy, which at least limits the extent of racemization, is to carry out the coupling reactions *at the highest possible concentration of the reactants* to ensure high coupling rates. This way the unimolecular, and hence concentration independent, racemization processes become less damaging.

A better approach to the conservation of chiral purity is offered by the *protecting groups* which are available for the blocking of the *α-amino* function. Already at the time of the introduction of the benzyloxycarbonyl group, its ability to protect against racemization during activation and coupling was noted and reported [67]. This unusual power to prevent the loss of chiral purity is absent from simple *N*-acyl groups such as the formyl, acetyl, trifluoroacetyl or benzoyl group and present only to some extent in the phthalyl group. On the other hand, several other amine protecting groups of the urethane type function equally well in this respect. Their ability to interfere with racemization was generally attributed to the lack of azlactone formation. The elimination of benzyl chloride and formation of *N*-carboxyanhydrides from *Z*-amino acid chlorides suggested [68] that
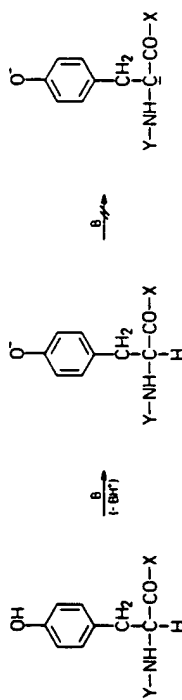
alkyloxycarbonylamino acids do not produce azlactones, the vulnerable intermediates. The formation of both the symmetrical anhydride and the 5(4H) oxazolone from benzyloxycarbonyl-L-valine and *tert*-butyloxy-carbonyl-L-valine on reaction with water soluble carbodiimides [15] demonstrates

the imperfectness of this rationale. It seems now that, while amino acids provided with a urethane-type amine protecting group do form azlactones, the latter retain their chiral integrity even under basic conditions. Thus, the former explanation requires revision, but the empirical rule that the benzyloxycarbonyl group and other urethane-type amine blocking groups prevent the racemization of the residues to which they are attached, remains valid. Notable exceptions are the blocked derivatives of S-alkyl-cysteine, O-alkylserine and β-cyanoalanine. Some other amine masking groups, e.g. the *p*-toluenesulfonyl and the *o*-nitrophenylsulfenyl group, are similarly protective in this respect.

The *influence of the activated residue* on the extent of racemization can be considerable but it is not always fully understood. The benzylic character of the chiral carbon atom in phenylglycine offers a simple explanation. It is less easy to interpret the somewhat reduced chiral stability of phenylalanine moieties, probably caused by the electron withdrawing effect of the aromatic nucleus even if it is separated by a carbon atom from the chiral center. On the other hand, tyrosine with a free phenolic hydroxyl was not racemized [69] in the coupling of Z-Val-Tyr via its azide in the presence of excess base, while the azide of Z-Val-His suffered considerable loss in chiral purity under similar conditions. An explanation might be found in the abstraction of a proton from the phenolic hydroxyl: the resulting anion interferes with the abstraction of a second hydrogen and therefore the chiral carbon does not become an anionic center:

$$Y\text{-}NH\text{-}\underset{H}{\overset{CH_2}{\underset{|}{\overset{|}{C}}}}\text{-}CO\text{-}X \;\xrightarrow[(\text{-}B\text{-}H^+)]{B}\; Y\text{-}NH\text{-}\underset{H}{\overset{CH_2}{\underset{|}{\overset{|}{C}}}}\text{-}CO\text{-}X \;\xrightarrow{B} \; Y\text{-}NH\text{-}\underset{H}{\overset{CH_2}{\underset{|}{\overset{|}{C}}}}\text{-}CO\text{-}X$$

In general, formation of dianions requires stronger bases than those used in peptide synthesis.

Racemization of activated valine and isoleucine residues occurs [70] in peptide synthesis. The electron release by the branched side chain s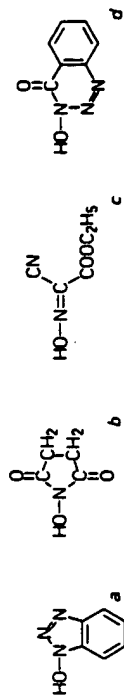hould destabilize the anion which has to be assumed in base catalyzed racemization processes and thus an alternative rationale must be found. The known assistance of bulky substituents in cyclization reactions might contribute to the formation of cyclic intermediates, e.g. azlactones, which play a role in the process of racemization. It is equally possible, perhaps even more likely, that, because of steric hindrance caused by bulky side chains, the coupling reactions proceed rather slowly and this allows more time for the progress of racemization. Chiral integrity is affected also by the residue(s) which precede the activated C-terminal amino acid in a peptide and also by the bulkiness of the N-terminal amino acid in the amino component [70]. The sequence dependence of racemization received, so far, only limited attention [71] and clearly requires further systematic studies.

Racemization of the C-terminal residue of amino components with a free C-terminal carboxyl was an unexpected discovery [72]. This side reaction, which is enhanced by 1-hydroxybenzotriazole and suppressed by N-hydroxysuccinimide, is probably due to the transient activation of the unprotected carboxyl group through interaction with the acylating agent:

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}X \;+\; H_2N\text{-}CHR'\text{-}\overset{O}{\overset{\|}{C}}\text{-}N\text{-}CHR''\text{-}\overset{O}{\overset{\|}{C}}\text{-}N\text{-}CHR'''\text{-}COOH \;\longrightarrow\; R\text{-}C\text{-}N\text{-}C\text{-}N\text{-}C\text{-}N\text{-}C\text{-}O\text{-}C$$

One of the most powerful methods for the preservation of chiral integrity is the use of *additives* or, perhaps more appropriately, of *auxiliary nucleophiles*. These can reduce the lifetime of overactivated, racemization-prone intermediates, such as O-acyl-isoureas. Also, the commonly applied additives have acidic hydrogens and thus can provide a proton which is more readily abstracted by bases than the proton from a chiral center. The best results reported so far were achieved with 1-hydroxybenzotriazole [73] (a), N-hydroxysuccinimide [74, 75] (b), 2-hydroxyiminocyanoacetic acid ethyl ester [76] (c) and particularly with 3-hydroxy-1,2,3-benzotriazine-4-one [77] (d).
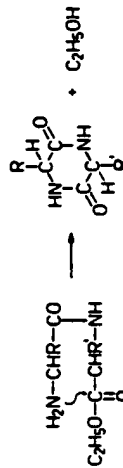
These racemization suppressing agents and several other potentially useful additives were compared by Izdebski [78].

From the foregoing discussion it is obvious that the extent of base catalyzed racemization is determined by a whole series of factors. An assessment of each of these in every coupling reaction is a demanding task and the results obtained so far are probably not entirely satisfactory since not all the influences are known, or at least not well enough to allow a quantitation of their contributions. Therefore, until the advent of truly racemization-free coupling methods, conservation of chiral integrity requires optimization in the choice of reagents, protecting groups, solvents, etc. Methods of activation which involve reactive intermediates containing a basic center should be used with caution. Overactivation, polar solvents should be avoided. The remaining choices are, however, not always conducive to an efficient formation of peptide bonds. Also, the selection of solvents is severely limited by the solubility of the intermediates. Hence, more weight has to be placed on the factors which provide some options and allow judicious decisions. For instance, the use of urethane-type amine protecting groups, attached to an amino acid rather than to a peptide, can greatly reduce the risk of racemization and the latter can be further diminished by avoiding the presence of tertiary bases in the reaction mixtures during activation and coupling. Last, but not least, the addition of well tested auxiliary nucleophiles creates conditions which no longer imperil chiral purity.

1.2 Undesired Cyclization

Dipeptide esters readily cyclize to form *diketopiperazines*. Ring closure can take place spontaneously because the thermodynamic stability of the six-membered ring overcomes the energy barrier in the formation of a *cis*-peptide bond, but the reaction is accelerated by bases, e.g. ammonia:

$$\underset{C_2H_5O\text{-}C\text{-}CHR'\text{-}NH}{\overset{H_2N\text{-}CHR\text{-}CO}{}} \;\longrightarrow\; \text{(diketopiperazine)} \;+\; C_2H_5OH$$

In solid phase peptide synthesis [79], where frequently polymer bound benzyl esters are present, this side reaction can cause some premature cleavage of the chain from the insoluble support [80–83]:

benzyloxycarbonyl groups can be carried out by catalytic hydrogenation in the presence of organic bases [240]; under the same conditions, benzyl ethers are not cleaved [241]. Peptides which provide multiple ligands for palladium, e.g. compounds with more than one methionine residue, resist hydrogenation even in the presence of base. Forced conditions, e.g. catalytic reduction for prolonged periods of time, result in desulfurization and formation of α-aminobutyric acid residues [242]. Reduction with sodium in liquid ammonia remains a viable choice, but excess sodium demethylates the methionine side chain [243].

Oxidation of the thioether to a sulfoxide occurs during the operations of peptide synthesis or during purification, but can be prevented by working in an inert atmosphere. Fortunately, oxidation to the sulfoxide is reversible. A mild treatment with thiols will reduce a sulfoxide to the thioether. Sulfones cannot be reduced under mild conditions, but they also do not form from thioethers unless powerful oxidizing agents are used.

Alkylation of the sulfur atom in the methionine side chain readily occurs during the removal of blocking groups by acidolysis [111, 119]. Some alkylations are easily reversed; e.g. S-tert-butyl sulfonium salts decompose on standing or on warming with the regeneration of the thioether [121]. Alkylation by the benzyl group is a more serious side reaction because S-benzylmethionine (salts) give rise to a variety of products [244], among them S-benzylhomocysteine. Therefore, in reactions where alkylating agents are generated the thioether should be kept intact with the aid of scavengers. Alternatively the methionine side chain can be protected by oxidation to the sulfoxide [123] or by reversible alkylation with methyl p-toluenesulfonate [124]. Alkylation by chloromethyl groups of polymeric supports should be avoided.

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The
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of Peptides

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HN——CHR

(25),NTA

case), and the progressive accumulation of over-reaction products limits the extent to which repetitive approach can be taken without difficult purification problems. The thio-analogues or NTAs (25) have also been used for peptide synthesis.[65] They are less prone to yield over-reaction products than NCAs, as the thiocarbamic acids produced by their aminolysis are less fragile. They are preferred in the special cases of glycine and histidine (the NCAs of which are especially subject to troublesome side-reactions), but unfortunately they are not secure against racemization.

5.1.2 Racemization*

Consider the synthesis of an all-L peptide comprising n chiral residues, from optically pure α-amino acids. If the operations needed for the incorporation of each residue result in conversion of a small fraction f of each residue to the D-form, and the epimers are carried through without separation, then the end product will consist of the required all-L peptide and a blend of other peptides in approximate proportions $(1-fn):fn$. For a synthesis of a 50-residue peptide in which 1% D-residue formation takes place at each stage, only half the final product will have the required all-L stereochemistry. The other 50% will consist mainly of about 1% each of all the 50 possible epimers with one D-residue. This will in general pose a prohibitive purification problem, and racemization in peptide synthesis has therefore been closely studied, with a view to defining the conditions under which it is minimal.[66-68]

Except for special cases (e.g., synthesis with N-methylamino acids: see Section 6.2.1), racemization is an almost exclusively base-induced side-reaction, and in practice is only a matter for serious concern at the activation and coupling stages of a synthesis. There are two important mechanisms.

---

*This term is used in peptide chemistry in a loose way which not only covers the strict sense as defined in most general organic chemistry texts (conversion of an enantiomer to a mixture of enantiomers), but also embraces partial epimerisation, whereby there is loss of chiral integrity at one out of two or more chiral centres, resulting in the formation of a mixture of epimers (i.e. diastereoisomers differing at one chiral centre).

Scheme 5.30.

Solvent envelope
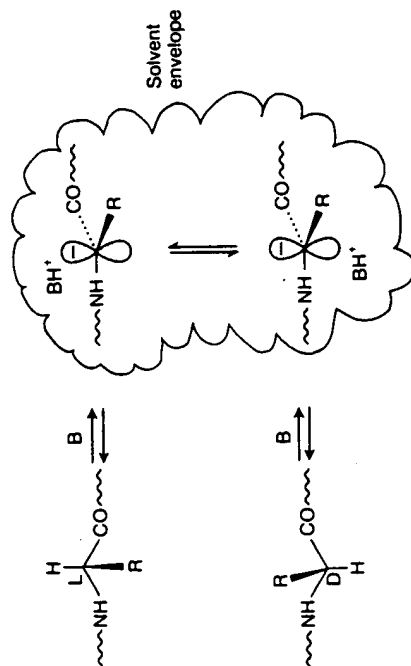
Scheme 5.31.

5.1.2.1 Direct enolization

Deprotonation at the α-carbon of an α-amino-acid residue results in racemization, because the carbanion intermediate can reprotonate on either side (Scheme 5.30). This has been called the 'direct exchange' mechanism, an inappropriate expression, because under some circumstances[69] racemization is much faster than exchange with the proton pool, implying that an ion pair is formed in which the ions are jostled about by solvent molecules and change their relative orientations without being divorced from each other, so that reprotonation can return the original proton to either side of the chiral centre ('isoracemization': Scheme 5.31). The rate of racemization by direct enolization depends on the catalysing base, the solvent, and the electron-withdrawing effects of the groups P,R, and X around the chiral centre (26). When X=NH-, O-alkyl, or O⁻, it is in most cases negligible,

and basic deprotection procedures other than saponification (see Section 4.1.1) are generally completely safe. During activation and coupling, the risk is rather more significant, but the danger is over once coupling is complete. Racemization is fastest with strongly electron-withdrawing groups X (i.e. most good leaving groups), and unhindered strong bases in dipolar aprotic solvents like DMSO and DMF. Inessential exposure to strong bases is clearly to be avoided, but the best way of responding to the other factors is not so obvious, because what matters is not the rate of racemization *per se*, but the balance between the rates of racemization and coupling, and this is much more difficult to make reliable generalizations about. Fortunately, with the exception of (a) a few special amino acids (α-arylglycines present quite a serious problem), and (b) couplings which are inordinately slow, the amount of racemization which actually takes place by this pathway is very slight indeed.

5.1.2.2 The oxazolone mechanism

Activated acylamino acids and peptides cyclize under the influence of base to give oxazolones [27, strictly '5(4H)-oxazolones', formerly '2-oxazolin-5-ones', archaically 'azlactones']. The oxazolones so formed are themselves activated towards aminolysis, and reaction with amino components leads ultimately to peptides, but since their racemization via stabilized anions is usually fast compared to the rate of peptide bond formation, any peptide thus produced is largely racemized (Scheme 5.32). Oxazolones are actually useful (e.g., references 70 and 71; see Scheme 6.31) for the activation of α-dialkyl-α-amino acid residues, where the question of racemization by base

Scheme 5.32. Conditions: basic.

(26)

(27)

does not arise because there is no hydrogen at the α-carbon. When the amino-nitrogen of the activated residue is acylated with a simple acyl group (acetyl, benzoyl, etc.), or with a peptide chain, cyclization to the oxazolone occurs easily with most good leaving groups X, and gross or even complete racemization may ensue. But oxazolone formation is not so facile when the acyl substituent is an alkoxycarbonyl protecting group. Indeed, the process was held to be impossible until 1977.[72] Furthermore, the alkoxyoxazolones 28 are both less easily racemized and more easily aminolysed than are the oxazolones 27 derived from simple acylamino acids. The activation of ordinary Z, Boc and Fmoc amino acids, etc., and their coupling with amino components is consequently not attended by the danger of racemization under normal conditions. This is a pivotal fact on which much of modern peptide synthesis turns. The reason for the contrast between, e.g., Z and benzoyl amino acids has not been fully explained, but a major factor is probably the lower acidity of BzlOCONH- compared to PhCONH-. In Scheme 5.32, the ring closure is shown as a specific base catalysed process, which has been demonstrated to be so in one set of circumstances.[72] It might be a concerted general base catalysed process under other conditions, but in either case lowering the acidity of the NH would be expected to diminish the rate of oxazolone formation.

A mechanism for oxazolone formation which does not require base assistance is also possible (Scheme 5.33), but ordinary amino acid derivatives do not cyclize this way except under very vigorous activation. *N*-Methyl-α-amino acid derivatives do, however, and give optically labile oxazolonium cations (see Scheme 6.29) even under normal activation conditions:[74] base catalysis is impossible because there is no NH for it to operate through, so cyclization can only occur by attack of the neutral amide oxygen on the activated carbonyl, which is easier with -CONMe- than -CONH- because

(28)

Scheme 5.33.

(29)

of electron release by the methyl group. Conformational restraints fortunately prevent this happening with proline derivatives, under all except the most extreme conditions.

Whether or not oxazolone-mediated racemization accompanies the activation and coupling of susceptible protected peptide acids depends on the leaving group. It does not seem to do so to a significant extent with acyl azide intermediates, in model systems at least, perhaps because these owe their aminolytic reactivity to intramolecular general base catalysis (see Section 5.1.1.2), to which oxazolone formation is indifferent because the nucleophile bears no hydrogen. And the risk is also small with the DCCI-HOBt procedure (see Section 5.1.1.4), because HOBt rapidly intercepts the activated species which might otherwise degenerate into oxazolones. The reactive HOBt ester intermediate favours aminolysis over oxazolone formation, possibly in part for the same reason as suggested for azides.

With activated protected peptides, the direct enolization and oxazolone mechanisms both provide pathways for the racemization of the carboxy-terminal residue. The formation of an oxazolone also threatens the chiral integrity of the penultimate residue, because the carbanion 29 is stabilised. Racemization at that residue has been observed at moderate levels in model experiments (e.g., reference 75), but has not so far been recognized as a real problem in actual syntheses, except when deliberate oxazolone formation is used to drive the coupling of carboxy components terminating in -XaaAibOH.[71]

5.2 The use of enzymes

This book is concerned with the chemical synthesis of peptides, so the inclusion of enzymic methods may raise a few eyebrows, but the use of enzymes as reagents in preparative organic chemistry,[76] without special homage to their biological origin, is burgeoning. A few remarks on enzymatic peptide bond formation[77,78] therefore seem called for.

Nature provides a wide range of proteolytic enzymes which can in principle be perverted to catalyse peptide bond formation by manipulating the conditions. There are two strategies for doing this. The first is dependent on thermodynamic control, the equilibrium in Scheme 5.34 (which

$$R'CO_2H + H_2NR^2 \rightleftharpoons R'CONHR^2 + H_2O$$

Scheme 5.34.

$$R'CO-X + H-Enz \longrightarrow R'CO-Enz$$

with $H_2O \longrightarrow R'CO_2H + H-Enz$

with $R^2NH_2 \longrightarrow R'CONHR^2 + H-Enz$

Scheme 5.35.

ZAspOH + HPheOMe $\xrightarrow{i}$ ZAspPheOMe $\xrightarrow{ii}$ HAspPheOMe

(30) Aspartame

Scheme 5.36. Conditions: i, 2 equiv. HPheOMe/pH7/thermolysin (the dipeptide salt precipitates); ii, HCl, then catalytic transfer hydrogenolysis with $HCO_2NH_4/Pd(C)/MeOH$.

favours hydrolysis overwhelmingly under normal conditions) being somehow displaced in favour of peptide bond formation. This can be achieved by employing protecting groups which will ensure precipitation of the peptide, or by using biphasic systems so that the peptide passes out of the aqueous phase into an organic solvent as it is formed, or by using water-miscible organic solvents which perturb the dissociation constants of the components and shift the balance of the equilibrium. The second strategy exerts kinetic control by arranging for an amino component nucleophile to compete with water for an acyl-enzyme intermediate (Scheme 5.35). The advantages of an enzymatic synthesis are the mild conditions, freedom from racemization and the need for side-chain protection, the possibility of using immobilized enzyme technology[79,80] with catalyst recovery, and the scope for industrial scale-up. Many examples have been reported. The synthesis[81] of the synthetic sweetener aspartame (30) is one of particular interest which has been developed for commercial application, and is also simple enough to be an undergraduate exercise[82] (Scheme 5.36). There are disadvantages, however. With peptides longer than dipeptides, there is the danger that while the protease is being persuaded to work backwards in creating a peptide bond at one point, it will remember the purpose for which evolution devised it and dismantle another somewhere else. No new case can be treated as

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of   :
   Gautvik et al.   :
                        : Group Art Unit: 1812

Serial No. 08/340,664   :

Filed: November 16, 1994   : Examiner: L. Spector

For:PRODUCTION OF HUMAN PARATHYROID   : Date:March 7, 1996
    HORMONE FROM MICROORGANISMS   :
_____X

Assistant Commissioner for Patents
Washington, D. C. 20231

EXTENSION PETITION
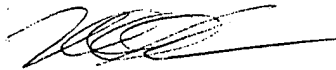
Sir:

The undersigned attorney respectfully petitions for a **three** month extension of time to reset the deadline for response to the Office Action in the above-identified application from December 8, 1995, to and including March 8, 1996. Applicant's **Reply and Amendment** is enclosed herewith.

A check in the amount of **$900.00** is enclosed. In the event that there are any fees due and owing, the Examiner is authorized to charge our Deposit Account No. 12-1095.

Respectfully submitted,

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of Gautvik et al.	:
	: Group Art Unit: 1812
U.S. Serial No.: 08/340,664	:
	: Examiner: L. Spector
Filed: November 16, 1994	:
	: Dated: March 7, 1996
For: PRODCUTION OF HUMAN PARATHYROID	:
HORMONE FROM MICROORGANISMS	:
X	

Assistant Commissioner For Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application. The fee has been calculated as shown below.

CLAIMS AS AMENDED

	(1) CLAIMS REMAINING AFTER AMNDT.		(2) HIGHEST NO.· PREVIOUSLY PAID FOR		(3) PRESENT EXTRA		RATE	ADD'L FEE
TOTAL CLAIMS	* 21	MINUS	** 20	=	1	x	$ 22	= $ 22.00
INDEP. CLAIMS	* 8	MINUS	*** 9	=	0	x	$ 78	= $ 0.00

FEE FOR FIRST PRESENTATION OF
MULTIPLE DEPENDENT CLAIM(S) $250 = $ 0.00

TOTAL ADDITIONAL FEE
FOR THIS AMENDMENT................................ $ 22.00

  *   If the entry in col. 1 is less than entry in col. 2, write "0" in col. 3.
 **   If the entry in col. 2 is less than 20, write "20" in col. 2.
***   If the entry in col. 2 is less than 3, write "3" in col. 2.

1.   ( )   No additional fee is required.
2.   (X)   A check in the amount of $22.00 is attached.
3.   ( )   Charge $_____ to Deposit Account No. 12-1095. A
                 duplicate copy of this sheet is enclosed.
4.   (X)   Please charge any additional fees or credit overpayment to Deposit
                 Account No. 12-1095. A duplicate copy of this sheet is enclosed.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of   :
    Gautvik et al.   :
                             :   Group Art Unit: 1812

Serial No. 08/340,664   :

Filed: November 16, 1994   :   Examiner: L. Spector

For: PRODUCTION OF HUMAN   :
    PARATHYROID HORMONE FROM   :   Date: March 7, 1996
    MICROORGANISMS   :
                             X

Assistant Commissioner for Patents
Washington, D.C. 20231

REPLY AND AMENDMENT

In complete response to the Official Action mailed September 8, 1995, please amend the application identified in the caption as follows:

IN THE CLAIMS

Please amend the claims as follows:

1.(Amended) Essentially pure , intact, recombinant hPTH.

Please cancel claims 2-5, 11, 13 and 15 without prejudice or disclaimer.

Please add the following claims:

21. The essentially pure, intact, recombinant hPTH of claim 1 wherein said hPTH possesses biological activity substantially equivalent to naturally occurring hPTH.

22. The essentially pure, intact, recombinant hPTH of claim 1 wherein said hPTH exhibits a maximal response which is greater than that which can be achieved by synthetic hPTH.

23. An essentially pure, intact hPTH obtained by expressing hPTH in a genetically engineered microorganism and purifying said hPTH.

24. The essentially pure, intact hPTH of claim 23, wherein said hPTH possess biological activity essentially equivalent to naturally occurring hPTH.

25. An essentially pure, intact hPTH of claim 23, wherein said genetically engineered microorganism is *E. coli*.

26. The essentially pure, intact hPTH of claim 23, wherein the genetically engineered microorganism is yeast.

27. An intact, recombinant hPTH which is fully active in an adenylate cyclase assay.

28. The intact, recombinant hPTH of Claim 27 wherein said hPTH is essentially pure.

IN THE TITLE

Please delete the current title "Production of Human Parathyroid Hormone From Microorganisms" and insert in place thereof, --Human Parathyroid Hormone.-- .

IN THE SPECIFICATION

Please amend the specification as follows:

On Page 1, please delete line 20 beginning with the word "This" through line 23, ending with the word "abandoned."

On Page 1, line 6, after "1993", insert -- now U.S. Patent No. 5,420,242 issued May 30, 1995--.

On Page 1, line 7, after "1993", insert --,now abandoned--.

On Page 8, line 25, please delete "Figure 8. Analysis" an insert --Figures 8a-8c show analysis--.

On Page 9, line 1, please delete "Figure 9. Purification" and insert in place thereof --Figures 9A - 9D show the purification -- .

On Page 9, line 28, please delete "Figure 13. Purity" and insert in place thereof --Figures 13A and B show the purity--.

<div align="center"><u>Remarks</u></div>

Entry of the foregoing and reexamination and reconsideration of the above captioned application, as amended, pursuant to and consistent with 37 C.F.R. § 1.112, and in light of the remarks which follow, are respectfully requested.

As a preliminary matter, the undersigned and Mr. Bruce Sales, Applicants' legal representatives, wish to thank Dr. Spector for the courtesies extended by her during an interview in her office on February 21, 1996. During that interview, the undersigned explained the deficiencies of the isolation and synthetic techniques described in the art identified by the Examiner in the Official Action. The undersigned also presented evidence in the form of photographs of electrophoretic gels showing the difference in purity

between recombinant hPTH produced in accordance with the present invention and synthetically produced materials. The Examiner was also shown a copy of a 1994 article in the refereed journal *Peptides* showing direct *in vitro* and *in vivo* comparative evidence between the recombinant hPTH of the present invention and the best synthetically produced material available today.

The Examiner acknowledged that such evidence was persuasive of differences between the claimed subject matter and the art of record.

Applicants also provided the Examiner with a copy of some draft claims and requested the Examiner's comments regarding same. The Examiner will note that in the draft claims presented during the interview, claim 1 was canceled in favor of claim 3. However, in the amendment proffered hereby, claim 1 is retained while claim 3 is canceled. The reason for this change is that the term "essentially pure" recited in claim 1 finds literal support in the specification at least at page 7, line 25 and is defined in the paragraph preceding that line. The term also finds implicit and inherent support throughout the specification, and in particular, Example 8, pg. 19, lines 7-10, pg. 5, lines 1-6, pg. 3, lines 17-24, pg. 4, lines 15-19, pg. 14, lines 9-14 and pg. 34, line 20 through pg. 35, line 5 to cite but a few passages. Applicants believe that the terms "substantially pure" and "essentially pure" are interchangeable in the context of the present invention. Therefore, Applicants have opted to elect a claim which finds literal support within the specification. This is consistent with comments made by the Examiner during the interview regarding identifying support for claimed terms.

Consistent with the foregoing, claims 23, 24, 25 and 26 have also been changed from "substantially" to "essentially". Literal support for newly added claims 27 and 28, not presented at the interview, can be found in the specification at page 7, lines 32-35. Again, however, implicit support is found throughout the specification, and in particular, at Example 8, pg. 19, lines 7-10, pg. 5, lines 1-6, pg. 3, lines 17-24, pg. 4, lines 15-19, pg. 14, lines 9-14 and pg. 34, line 20 through pg. 35, line 5. Applicants also respectfully submit that claims 23, 24, 25 and 26 are supported

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throughout in the specification as a whole, and in particular at pg. 7 lines 32-35. Claims 21, 22 and 24 now depend from claim 1.

Turning to the Official Action, Applicants first acknowledge the Examiner's designation of the Restriction Requirement as being "Final".

The specification was objected to 37 C.F.R. § 1.75(d)(1) and M.P.E.P. § 608.01(l). The Examiner indicated that correction of the specification was required as there was no antecedent basis in the specification for the claim recitations 90% or 95% purity. Applicants respectfully submit that there is literal support for these terms. Nonetheless, as the claims at issue have been canceled hereby, the objection is believed to be moot. Should the Examiner consider it helpful for Applicants to identify where in the specification such literal language can be found, Applicants are willing to do so.

The Examiner also objected to the title of the invention as not being sufficiently descriptive. Accordingly, Applicants have deleted the title and proffered a new title which identifies the subject matter of the claimed invention. Should the Examiner require additional corrections, Applicants would appreciate the Examiner's assistance in drafting a title which is sufficiently descriptive.

The Examiner has asked that the status of all applications to which references are made in the first paragraph of the application be updated and has suggested that the information on page 1, lines 20-23 is duplicative of the first paragraph. The Examiner is correct. The duplicative passages have been deleted. The status of the applications identified in the first paragraph on page 1 has been updated.

The Examiner objected to certain of the Brief Descriptions of the Drawings noting that they should reflect each individual panel of each figure. Appropriate corrections have been made. Should the Examiner require any additional change thereto, Applicants would request the Examiner's assistance in identifying specific language which would be considered acceptable.

Applicants also acknowledge the Examiner's objections to Figs. 6, 7 and 10. Applicants will offer such corrections

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as are necessary once there has been an indication of allowable subject matter. Should this present a problem, Applicants respectfully request that the Examiner so inform the undersigned and expedited steps will be taken to address the figures.

Claims 1-5, 11, 13 and 15 stand rejected pursuant to the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 6 of U.S. Patent No. 5,010,010. Applicants will not traverse the rejection and instead will offer, once allowable subject matter is indicated, a properly executed terminal disclaimer. U.S. Patent No. 5,010,010 is commonly assigned with this application. Accordingly, a terminal disclaimer should, at least provisionally, render moot the double patenting rejection. Should the Examiner require the submission of a terminal disclaimer prior to an indication of allowable subject matter, Applicants will attempt to expedite same. The Examiner's indulgence with regard to the filing of such formal papers is appreciated since the inventors and assignees of the application reside outside the United States.

The Official Action contained a number of specific objections and rejections pursuant to 35 U.S.C. § 112, 1st and 2d paragraphs. Applicants believe that the claims, as amended, render moot those objections and rejections. Specifically, Applicants have amended the application so that it no longer claims "a synthetic" peptide and no longer claims both an "essentially pure" and a "substantially pure" peptide. Applicants have also amended the product-by-process claims so that they now explicitly require a purification step. Again, should the Examiner have any specific questions with regard to any of the language used in the claims, she should feel free to contact the undersigned.

The claims, prior to amendment, were rejected pursuant to 35 U.S.C. § 102(b) over *Kumagaye et al., Kimura et al., Fairwell et al.* and *Brewer et al.* The same claims were rejected over the same four references, when considered pursuant to 35 U.S.C. § 103. Applicants respectfully traverse these rejections as applied to the amended claims.

As discussed during the interview, and for the convenience of the Examiner, Applicants will keep their

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comments with regard to the prior art to a minimum. Instead, Applicants respectfully request that the Examiner consider the detailed comments of Dr. John Maggio and Dr. Kaare M. Gautvik contained in their concurrently filed declarations. As appropriate, Applicants have referenced the pertinent paragraphs of the declarations to their discussion. Of course, if the Examiner has any questions regarding the declarations or requires further comments of Applicants, she should contact the undersigned.

Brewer et al. relate to the isolation of, primarily, the 34 amino acid N-terminal region of hPTH. Brewer et al. do not teach a recombinant product. (Maggio Decl. ¶ 9). Moreover, Fig. 1 of Brewer et al. contain three errors in the amino acid of the first 34 amino acids. (Maggio Decl. ¶ 9). This calls into question whether or not Brewer et al. actually produced an intact hPTH. (Maggio Decl. ¶ 9). The purification technique used by Brewer et al. was gel filtration followed by ion exchange chromatography.

Kimura et al., a later-dated reference cited by the Examiner, clearly demonstrates that Brewer et al.'s purification techniques were inadequate. In Fig. 2, Kimura et al. show a chromatogram of hPTH from Brewer et al. purified by gel filtration and ion exchange chromatography. (Maggio Decl. ¶ 9). Impurities are clearly evident. (Maggio Decl. ¶ 9). To obtain their own purification, Kimura et al. employed an additional reverse phase-high pressure liquid chromatography (RP-HPLC) step not employed by Brewer et al. The need for the RP-HPLC step further evidences the inadequacies of the Brewer et al. protocol. (Maggio Decl. ¶ 9).

Fairwell et al. suffers from many of the same problems as Brewer et al. (Maggio Decl. ¶ 10). Like Brewer et al., Fairwell et al. employed a separation protocol based on gel filtration followed by ion exchange chromatography. Therefore, Applicants' prior comments with regard to Kimura et al. are equally cogent here. (Maggio Decl. ¶ 10). Additionally, Fairwell et al. produced a point mutant of hPTH; Asp in position 76 instead of Asn. (Maggio Decl. ¶ 10).

Kimura et al. do not teach essentially pure hPTH. In Kumagaye et al., which is a later paper by the Kimura et al.

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group, the investigators acknowledged that "[t]oday, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC [the procedure used by *Kimura et al.*] is not sufficient to obtain homogeneous products." *Kumagaye et al.* at 330. (Emphasis added) (Maggio Decl. ¶ 11). In fact, *Kimura et al.* acknowledge that they were not able to resolve native hPTH from its Asp^{76} point mutant. *Kimura et al.* at 498.

Finally, *Kumagaye et al.* does not provide sufficient basis for one of ordinary skill in the art to conclude that essentially pure hPTH was produced. (Maggio Decl. ¶ 12). Although *Kumagaye et al.* appears to have resolved hPTH from the Asp^{76} point mutant, purity is not demonstrated. The results only mean that the *Kumagaye et al.* material was free of the Asp^{76} point mutation. (Maggio Decl. ¶ 12). No other inference can be drawn. In fact, if an inference were to be drawn at all, the inference would be that the resulting material was not essentially pure because of the well-known problems associated with solid phase chemistry. (Maggio Decl. ¶¶ 12, 17).

During the interview, the Examiner acknowledged what the art recognized; namely, that there are significant impurity problems with synthetic, solid phase protocols such as those disclosed in *Kumagaye et al.*, *Kimura et al.* and *Fairwell et al.* Premature chain termination, omitted coupling steps, and double coupling steps are widely known and frequently occurring problems. (Maggio Decl. ¶ 15). See also *Fairwell et al.* at page 2691. In addition, synthetic protocols, including these employing the use of BOC chemistry, are known to produce racemization. (Maggio Decl. ¶ 14). For example, *Kimura et al.* describe possible contamination by a $D\text{-}Glu^{22}$ containing peptide. The incidence of these types of impurities increases exponentially with the length of the peptide and a peptide of 84 amino acids in length, such as hPTH is considered long, even by today's standards. (Maggio Decl. ¶¶ 14, 15 and 16). Therefore, those of ordinary skill in the art would expect the presence of impurities based on the type of solid phase chemistry employed in the references cited. (Maggio Decl. ¶ 17).

Moreover, as those of ordinary skill in the art appreciate, unless all of the impurities produced possess a sufficient charge differential, they <u>cannot</u> be separated from intact hPTH using of the techniques described in the art of record. (Maggio Decl. ¶ 16). Consequently, one of ordinary skill in the art would be prone to speculate that the material produced in accordance with, for example, *Kumagaye et al.*, would contain many of these impurities, impurities which would co-elute with hPTH. (Maggio Decl. ¶ 17).

For the reasons just explained, Applicants believe that it would be improper for one to conclude that any of the references applied in the aforementioned Official Action teach or suggest the production of essentially pure hPTH. On that basis alone, Applicants believe that the various prior art rejections are fully met. Nonetheless, during the interview, the Examiner expressed her opinion that despite the references' shortcomings, Applicants would still need to establish that the recombinant hPTH of the present invention was superior, in terms of its claimed attributes, when compared to synthetically produced material.

The evidence necessary to establish the superiority of the present invention can be found in the exhibits attached to Dr. Gautvik's declaration and in the comments of Drs. Gautvik and Maggio. Again, for the convenience of the Examiner, Applicants will not belabor the record reexplaining that which is eloquently set forth by the declarants. Instead, the undersigned will merely summarize some of the more noteworthy points of the declarations. Should the Examiner feel that she would benefit from further explanation, please contact the undersigned.

All of the electrophoretic gels and data presented in the refereed *Peptides* paper, attached to Dr. Gautvik's declaration, were obtained using hPTH material produced in the mid- to late 1980s following the exact protocols disclosed in the above-captioned application. (Gautvik Decl. ¶ 5). The only purification steps used were those described in the application itself. (Gautvik Decl. ¶¶ 5, 10). Applicants respectfully submit that the continuing viability of that batch of material, over the many years since its recombinant

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synthesis, speaks volumes with regard to its purity. (Gautvik Decl. ¶ 9).

As shown in Glossy 0 (Gautvik Decl. Ex. B), hPTH produced in accordance with the present invention migrated as a single bright band. (Lane 2, second from the left). Commercially available synthetic material from Sigma, shown in Lane 3 (third lane from left), includes two distinct bands of impurities of higher molecular weight than the hPTH of the present invention. (Gautvik Decl. ¶ 6; Maggio Decl. ¶ 20). The relative intensities and breadth of the bands of impurities are significant because each lane was originally loaded with an identical amount of peptide material. (Maggio Decl. ¶ 20).

Glossy III (Gautvik Decl. Ex. E) shows a comparison between electrophoretic gel recombinant hPTH and what is considered to be the best available synthetic material from the supplier Bachem. The gel distinctly shows a smear of lower molecular weight impurities. (Maggio Decl. ¶ 21; Gautvik Decl. ¶ 9). Additionally, Glossy II (Gautvik Decl. Ex. D) shows that even at a loading of 200 nanograms, very little Bachem material was discernible while strong, broad bands of recombinant material are clearly evident. (Maggio Decl. ¶ 22; Gautvik Decl. ¶ 8).

Applicants draw the Examiner's attention to the paper entitled "Differences in Binding Affinities of Human PTH(1-84) Do Not Alter Biological Potency; a Comparison Between Chemically Synthesized Hormone, Natural Mutant Forms", *Peptides* (1994) Vol. 15, No. 7, pgs. 1261-1265 ("the *Peptides* paper"), a copy of which is attached to Dr. Gautvik's declaration as Exhibit F. This work, undertaken by Dr. Gautvik and his coworkers, was published in 1994. This paper is significant because of the consistency of the results reported with the electrophoretic evidence just discussed. Moreover, as Dr. Maggio points out in his declaration, the paper is greatly pertinent because of its rigorous statistical treatment of the subject matter (provision of 95% confidence intervals and its repetitive testing in triplicate) and because of the variety of testing methods utilized. (Maggio Decl. ¶ 24). Coupled with the variety of methods reported in the specification of the above-captioned application, this article provides a high degree of confidence with regard to the correlation between the gels, the

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testing reported in the *Peptides* article and the testing reported in the application.

Fig. 1 of the *Peptides* paper shows the inhibition of radiolabeled [Tyr[36]] chicken PTHrP(1-36) amide by different hPTHs. As shown in the figure and as explained on page 1264, chemically synthesized hPTH had a calculated binding affinity (K_d) of 18nM while the recombinant hPTH from both yeast and *E. coli* had a significantly lower apparent K_d of 9.5nM. This represents an almost two fold difference between the binding affinity of the recombinant hPTH in accordance with the present invention when compared to the material tested -- the best available synthetic material from Bachem. (Maggio Decl. ¶ 24). The provision of 95% confidence intervals further enhances the high statistical significance of this data. (Maggio Decl. ¶ 24).

Biological activity also supports the purity of Applicant's hPTH. As shown in Fig. 2 and explained on page 1264 of the *Peptides* paper, the recombinant hPTH of the present invention has significantly higher biological activity compared to material produced by solid phase chemical synthesis. Specifically, the recombinant hormone exhibited an almost four fold increase in its ability to stimulate intracellular cAMP accumulation. The EC_{50} values for synthetic hormone were 1.5nM while the recombinant material exhibited an EC_{50} of only 5.7nM. (Maggio Decl. ¶ 25; Gautvik Decl. ¶ 12). Perhaps more importantly, and also as shown in Fig. 2, recombinant hPTH in accordance with the present invention achieved a higher maximal response or efficacy when compared to the best available synthetic material. (Maggio Decl. ¶ 26; Gautvik Decl. ¶ 12). This means that no amount of synthetic material could provide the same efficacy as a maximal dose of recombinant hPTH. (Maggio Decl. ¶ 26; Gautvik Decl. ¶ 12).

The *in vivo* evidence presented in the *Peptides* paper is equally compelling. For the sake of brevity, Applicants merely point to the hypercalcemic assay illustrated in Fig. 3 and discussed on page 1264. In order to achieve an effect almost equal to that of 2.0 micrograms of recombinant hPTH, a 2.7 micrograms dose of chemically synthesized hPTH was required. Thus, the synthetic hPTH had a 30% lower biological activity than the recombinant hPTH. This contrasts markedly

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with the maximal response achieved from a considerably lesser dosage of recombinant hPTH. (Maggio Decl. ¶¶ 26, 27).

As the foregoing data compellingly establish, the purity of the recombinant hPTH of the present invention is vastly superior to that of synthetic peptide. This is demonstrated by gels equally loaded with recombinant and synthetic hormone in which synthetically produced hormone had both impurities and significantly lower band intensities; by *in vitro* biological testing which showed that recombinant hPTH exhibited a two-fold increase in affinity, a four-fold increase in biological potency and a higher maximal response or efficacy than could be achieved by the synthetic peptide; and by *in vivo* testing of dosage levels and maximal response studies in which the recombinant hormone was constantly superior to the synthetic hormone.

The recombinant hPTH of the present invention is superior not only in terms of its quantity but also in terms of its qualities. (Maggio Decl. ¶ 27). Because the hPTH of the present invention is produced by recombinant technology, it does not suffer from many of the known disadvantages of synthetic production. Those of ordinary skill in the art would clearly understand this to be the case based on their knowledge of the shortcomings of solid phase synthesis and the fact that the cellular editing mechanisms available in microorganisms such as yeast and *E. coli* would all but eliminate the inherent impurities created during synthetic synthesis. (Maggio Decl. ¶ 17). Therefore, those of ordinary skill in the art would understand that the term "recombinant" describes not only a process by which the resulting hPTH peptide was produced, but also laudatory characteristics which describe the purity and the functional qualities of the resulting material. At the very least, when such a term modifies the term "essentially pure" it indicates the types of impurities that could not be present in the claimed hPTH.

In summary, Applicants believe that claim 1, as amended, has three specific points of novelty, each of which could independently support a conclusion of patentability. First, the claim recites that the hPTH material obtained must be "essentially pure" as that term is defined in the specification. The supporting evidence presented herein

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clearly establishes the overwhelming superiority of the purity of the hPTH material produced in the present invention versus the best available material produced by the techniques described in the art of record. Second, the hPTH produced must be "intact." "Intact" hPTH is structurally identical to the naturally occurring peptide; it is full length and identical in amino acid composition. Finally, the material is novel, and indeed, unobvious because it is produced recombinantly. The use of the term "recombinant" in the context of the present invention clearly provides additional information to those of ordinary skill in the art with regard to the nature and qualities of the hPTH claimed.

Claim 23 which relates to a product-by-process is also novel and unobvious for the same reasons. Novelty resides in the process used for the production and purification of hPTH as recited in the claims. Claim 27 as well as dependent claims 21, 22, 24 through 26 and 28 are also all novel and unobvious. It has been shown that the recombinant hPTH of the present invention is "fully active" in adenylate cyclase assay...." (Page 7, lines 33 and 34), (Gautvik Decl. ¶ 13). *See also* Example 8, pg. 19, lines 7-10, pg. 5, lines 1-6, pg. 3, lines 17-24, pg. 4, lines 15-19, pg. 14, lines 9-14 and pg. 34, line 20 through pg. 35, line 5. As Dr. Gautvik explains in his Declaration, the fact that hPTH exhibits full activity in this assay indicates that the hPTH of the present invention possesses a level of biological activity which is substantially equivalent to naturally occurring hPTH. The cAMP testing described in the *Peptides* paper only serves to confirm the statements made in the specification. (Gautvik Decl. ¶ 13).

Applicants believe that the claims, as amended, are both novel and unobvious and that the claims satisfy all of the requirements imposed by 35 U.S.C. § 112. Therefore, Applicants respectfully request the withdrawal of the current rejections and an indication of the allowability of the presently claimed invention.
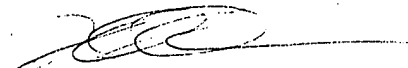
Should Examiner Spector have any questions with regard to the foregoing, she should feel free to contact the undersigned, at her convenience at 908-654-5000. Furthermore, should any fee be due and owing regarding this matter, the

Examiner is hereby authorized to charge Deposit Account No. 12-1095 therefor.

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order and such action is earnestly solicited.

Respectfully submitted,

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK

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CURRICULUM VITAE

Kaare M. Gautvik, professor dr.med.

Personal and marital status

Name: Kaare M. Gautvik
Home address: Bregnevn. 3, 0875 Oslo, Norway
Business address: Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112
                     Blindern, 0317 Oslo, Norway
Telephones: 47-22851055 (work); 47-22235137 (home)

Date and place of birth: 11th of December 1939 in Oslo.
Social Security No.: 111239.39311

Married to: Vigdis Teig Gautvik, date of birth: 24th of March 1947

Children: Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964
               Silja Marie Sakrisvold Gautvik, date of birth: 31th of March, 1973
               Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education

1.   August 1958-June 64, Medical School at the University of Oslo.
2.   1967-69 Courses in mathematics involving
           geometry, statistics and mathematical analysis.
3.   May 1970, Disputation for the medical doctor degree at
           the University of Oslo.
4.   1985, Specialist in medicine, in clinical chemistry and physiology.

Employment

1.   June 1964 - June 1965, working at Tromsø University Hospital at medical and surgical
     departments.
2.   July 1965 until December 1965, working as a general practitioner in Sjøvegan, Troms.
3.   One year military service as a major in The Norwegian Air Force, working mainly at the
     Norwegian Institute for Aviation and Space Medicine.
     From 1967, position as post-doctoral researcher at The Institute of Physiology, Uni-
     versity of Oslo.

5.   From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6.   Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973 - 89.
7.   From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8.   From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
        (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).

Post-doctoral training abroad

1.   For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2.   From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3.   1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4.   1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility

1.   One year teching in aviation medicine for medical personnel and pilots.
2.   I have given lectures and courses for medical students in following subjects: Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology. From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3.   Organized interfaculty advanced courses within molecular endocrinology.
4.   Lectures have been given in the following subjects at post-doctoral courses: Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5.   Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.
6.   Chief organizer of post graduate scientific courses for the Medical Fakulty at University of Oslo, 1986-1991.

7.   Organizer of international scientific meetings within the frame of the following societies:
      Acta Endocrinologica (European International Endocrine Society), The Scandinavian
      Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8.   Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9.   Invited as Symposium Lecturer at international meetings in physiology and
      endocrinology and molecular biology as exemplified below:

Examples of specially invited symposium lectures

1.   February, 1990: "Production of recombinant human parathyroid hormone in E.coli and
      Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M.
      Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.

2.   June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal
      transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.

3.   July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the
      regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary
      cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.

4.   July, 1990: Symposium lecture: "Successful cloning and production of human
      parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress
      on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held
      by cand.scient. Sjur Reppe).

5.   August, 1990: Symposium lecture: "Processing and stability of human parathyroid
      hormone produced in E.coli and S.crevisiae studied by in vitro mutagenesis". Work-
      shop/Symposium on site-directed mutagenesis and protein engineering, Tromsø,
      Norway.

6.   December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor
      G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone
      as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second
      International Conference on Medical Microbiology and Biotechnology Towards 2000,
      Guangzhou, China. (Did not attend as a protest against the punishment of the students
      rebellion in Peking).

7.   January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology
      Laboratory, ICI, Alderly Park, Maccelesfield SK10 4TJ,UK. Lecture entitled:
      "Synergistic effects of hormones and fatty acid on peroxisomal ß-oxydation, enzyme
      activities and mRNA levels".

8.   January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell,
      Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning

and expression of human parathyroid hormone in microorganisms".

9.   Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology
      Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine
      Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could
      not attend).

10.   May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to
      held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biol-
      ogy".

11.   January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and
      Cellular Toxicology, Harvard School of Public Health and Department of Biological
      Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA.
      Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of
      G-coupled hormones".

12.   August 1993: Invited by Norwegian Society of Charted Engineers, The Blindern
      Conference. Lecture entitled: "Experience from industrializing basal research".

13.   November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at
      "Graduate course in molecular endocrinology - a problem oriented approach". The
      lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".

14.   February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH,
      Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in
      microorganisms and animal cells with special reference to signal sequence efficacy and
      intracellular modifications".

15.   September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology,
      Madurai Kamaraj University, India, to held a lecture in the symposium "Gene
      expression systems", XVIth IUBMB, New Delhi. Lecture entitled: "Expression of
      human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a
      milk protein in transgenic mice".

16.   November 1994: Invited by Professor A. Taschjian Jr., Harvard School og Public
      Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and
      functional characteristics of the human TRH receptor cDNA and mapping of the gene".

17.   February 11-13, 1995: Cairns, Australia, Workshop on "Animal Models in the
      Prevention and Treatment of Osteopenia"

18.   February, 1995: Int. Meeting of Calcified Tissue Research, Melbourne, Australia.

Honorary lectures and prizes

1.   In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize
      and lecture was created by Professor Torgersen, the University of Oslo, who was one of
      the founders of the Society for Promotion of Cancer Research in Norway. Because he
      contributed with personal money, the prize and lecture had his name. The title of my
      lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone
      producing cancer".
2.   In 1984 I was given the international science prize called The Nordic Insulin Prize
      instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen.
      This prize is given within endocrinology and the candidate is chosen from all the countries
      in Northern Europe. The money was donated by the Insulin Laboratory now the company
      Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The
      prize was given for my studies regarding how hormones exerted their biological actions in
      target cells.
3.   The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists.
      This is a prize which is given to a scientist selected by this society for scientific merits
      obtained and again it was within the field of hormone structure and action.
4.   In 1987 I received a prize within biotechnology created by the Research Park at the
      University of Oslo, which at that time was called the Innovation Centre, University of
      Oslo.
5.   Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific
      prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska
      Institute, Sweden, in 1991. This was given based on my research with human parathyroid
      hormone in relation to its first cloning, expression and studies of actions.
6.   Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995,
      regarding cloning of hormone genes and their characterizations. Invited by Professor A.H.
      Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of
      Public Health and Department of Biological Chemistry and Molecular Pharmacology,
      Harvard Medical School, Boston, USA.

Referee activity

I am or have been working as referee for the following international journals:
                        Endocrinology
                        J. Expl. Cell Res.
                        Acta Physiol. Scand. (Kbh.)
                        Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.)
                        Eur. J. Clin. Invest.
                        Hormone Research
                        Acta Obstet. Gynecol. Scand.
                        Journal of Endocrinological Investigation
                        Eur. J. Biochem.
                        Experimental Cell Research
                        Scand. J. Gastroenterol.

Guidance for the academic doctor degree

1. Veterinary, dr.lic. Richard Tollman: "Parturition hypocalcaemia in cows". 1976. Oslo.

2. Dr.med. Trine Normann: "Medullary carcinoma of the thyroid. A morphological, clinical and experimental study". 1977. Oslo.

3. Dr.med. Egil Haug: "Prolactin and growth hormone secretion by rat pituitary cells in culture. Hormonal control and mechanism of action". 1978. Oslo.

4. Dr.med. Bjørn Klevmark: "Motility of the urinary bladder in cats during filling at physiological rates." Oslo. 1978.

5. Dr.odont. Torill Berg Ørstavik: "Glandular kallikreins. Origin and secretion in some exocrine organs of the rat". 1978. Oslo.

6. Dr.med. Arne Ekeland: "The role of calcitonin in fracture healing". 1981. Oslo.

7. Dr.philos. Kjersti Sletholt: "Calmodulin from rat anterior pituitary tumour cells and its biological significance". 1988. Oslo.

8. Dr.med. Trine Bjøro: "Regulation of prolactin secretion by hypothalamic hormone with special emphasize on vasoactive intestinal polypeptide (VIP)". 1988. Oslo.

9. Dr.scient. Øyvind Andersen: "Purification and characterization of salmonid prolactin". 1989. Oslo.

10. Dr.scient. Marianne Wright: "Biochemical studies of the pituitary receptor for thyro-thropin-releasing hormone. Cell surface receptor protein characterization, receptor mRNA isolation and cDNA library generation and screening". 1991. Oslo.
Dr.phil. Vendela Parrow: Signal transduction and gene regulation in cultured endocrine cells. 1991. Oslo.

11. Dr.med. Eyvind J. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.

12. Dr.philos. Ruth H. Paulssen: "G protein-coupled transmembrane signalling in prolactin-producing rat pituitary tumour cells". 1992. Oslo.

13. Dr.scient. Hilde Nebb Sørensen: "Actions of hormones and fatty acids on peroxisomal ß-oxidation enzyme activities and gene transcription." (Disputation 1993).

14. Dr.philos. Najma Kareem: "The use of protein engineering to study hormone processing and secretion in different host cell systems". (Disputation 1994).

15. Dr.philos. Ole Kristoffer Olstad: "Expression, purification and characterization of recombinant parathyroid hormone like peptides". (Disputation 1995).

16. Dr.med. Berit Mortensen: "The influence of vit. D₃ on bone remodelling: In vitro and in vivo studies of bone turnover in the normal and uraemic conditions". (Disputation 1995).

17. Dr.philos. Venke Skibeli: "Structural and functional aspects of Atlantic salmon growth hormone and prolactin". (Disputation 1996).

18. Dr.med. Erik Rokkones: "Expression of heterologous peptide hormone genes in cultured cells and in animals". (Disputation 1996).

19. Dr.philos. Sjur Reppe: "Secretion of heterologous proteins from the yeast Saccharomyces cerevisiae". (Disputation 1996).

Supervision of postgraduate candidates, thesis works:

I.   Cand.pharm. Per Wiik Johansen: "Regulation of prolactin and growth hormone
        secretion and synthesis by bromocriptine in rat anterior pituitary tumour cells".
        (Disputation 1996).
II.   Cand.scient. Vilborg Matre: "Cloning and expresjon of membrane receptors for
        hypothalamic hormones in exitable cells".
III.   Cand.scient. Hilde Hermansen Steineger: "Studies of regulatory gene-elements and
        transcription factores that mediate peroxisomal inducton and proliferation".
IV.   Cand.scient. Ole Petter Løseth: "Studies on hormonel bone remodulation in tissues and
        in animals".
V.   Cand.scient. Per Ivar Høvring: "Structure analysis and functional studies of cloned
        thyroliberin receptor and receptor isotypes".
VI.   Siv.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells".

Supervision of students' main degrees

1.   Cand.pharm. Åse Aulie: "The effect of somatostatin on cultures of growth hormone and
        prolactin producing cells." University of Oslo. 1979.
2.   Cand.pharm. Per Wiik Johansen: "The effects of bromocriptin on prolactin and growth
        hormone producing rat pituitary gland cells in culture." University of Oslo. 1981.
3.   Cand.pharm. Nina Lillegraven: "The significance of extracellular ion influence on the
        binding of thyroliberin to rat pituitary gland cells in culture." University of Oslo.
        1982.
4.   Stud.med. Eyvind I. Paulssen: "The effect of TRH and oestradiol on prolactin-synthesis
        in rat pituitary cells in culture." University of Oslo. 1983.
5.   Cand.pharm. Kari Furu and cand.pharm. Kirsten Kilvik: "The uptake mechanism for
        oestradiol in rat pituitary cells in culture." University of Oslo. 1984.
6.   Cand.pharm. Berit Taranrød Johansen: "Cloning of mRNA for rat prolactin". U
        niversity of Oslo. 1986.
7.   Cand.scient. Marianne Wright: "Characterization of surface proteins of GH-cells with
        special reference to the TRH receptor". University of Oslo. 1987.
8.   Cand.scient. Jenny Owe: "Binding and degradation of thyrotropin releasing hormone in
        hormone producing rat at pituitary cells in culture". University of Oslo. 1988.
9.   Cand.real. Grete Sørnes: "Effects of vitamin D on $^{45}Ca^{2+}$ efflux and prolactin produc-
        tion". University of Oslo, 1988.
10.   Cand.pharm. Siv Eriksen: "Development of an in solution mRNA hybridization test
        using antisense mRNA probes for prolactin". University of Oslo. 1988.
11.   Cand.real. Tom Skyrud: "Effects of human growth hormone and IGF-I on growth and
        clinical chemical plasma parameters". University of Oslo. 1988.
12.   Cand.pharm. Laila Northeim: "The inductive effect of tetradecyl-thio-acetic acid on
        peroxisomal ß-oxidation in 7800 C1 Morris hepatoma cells is stimulated by dexame-
        thasone and inhibited by insulin". University of Oslo. 1988.
13.   Dipl.ing. Kristin Austlid Taskén: Transfeksjonsstudier i Karpe- og rotte hypofyseceller.
        University of Trondheim (NTH)/University of Oslo. 1988
14.   Cand.real. Hilde Nebb Sørensen: "The mechanism of ^{3}H-Dexamethasoneuptake into

7800C₁ hepatoma cells in culture". University of Oslo. 1989.

15. Stud.real. Najma Kareem: "Secretion and processing of recombinant hPTH in E.coli. Significance of preprosequences". University of Oslo. 1989.

16. Stud.ing. Edith Rian: "Expression of parathormone-like peptides in tumour cells.

17. Cand.scient. Even Sollie: "Stability of peroxisomal ß-oxidation enzyme activity and mRNA levels". University of Oslo. 1993.

18. Stud.scient Åse-Karine Fjelheim: Cloning and characterization of the human thyrotropin releasing hormone. (1996).

Guest research workers from abroad

In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.

Member of comittees for the academic doctor degree in Norway and abroad.

1. Opponent at dr.med. Bjørn Biber's disputation at Physiological Institute, The University of Gothenburg, Sweden. The work represented gastrointestinal physiology. 1974.

2. First opponent at dr.med.vet. Knut Hove's disputation. The work represented the effect of insulin on the intermediate metabolism in ruminants and mammary gland physiology. University of Tromsø, Norway, 1978.

3. First opponent at siv.ing. Kirsten Sandvig's disputation on the work: "Interaction of the toxic lectins abrin, ricin, and modeccin with mammalian cells". The work includes biochemical examinations on absorbtion and effect of toxic lectins in cell cultures. University of Oslo, Norway, 1979.

4. Opponent at cand.real. Anne Sundby's disputation on the work: "Plasma testosterone in young bulls in relation to age, gonadotropin stimulation and rate of weight gain and some studies on testicular gonadotropin receptors". University of Oslo, Norway, 1982.

5. Opponent at siv.ing. Anders Sundan's disputation on the work: "Studies on the entry of modeccin, diphtheria toxin, ricin, and pseudomonas toxin into mammalian cells". University of Oslo, Norway, 1985.

6. Opponent at dr.med. Svein Dueland's disputation on the work: "Absorption and transport of vitamin D₃ and 25-hydroxy-vitamin D₃ in the rat". University of Oslo, Norway, 1986.

7. Opponent at dr.philos. Dagny Sandnes's disputation on the work: "Beta-adrenoceptors on rat hepatocytes and human mononuclear leucocytes, with special reference to quantitation and regulation". University of Oslo, Norway, 1988.

8. Opponent at dr.med. Øyvind Sverre Bruland's disputation on the work: "Preparation and properties of two novel highly specific antisarcoma monoclonal antibodies and their application in the characterization and diagnosis of human sarcomas". University of Oslo, Norway, 1989

9. Opponent at dr.med. Pål Wiik's disputation on the work: "Vasoactive intestinal peptide as a modulator in the neuro-immune axis; the influence of stress". Norwegian Defence Research Establishment, Norway, 1989.

10. Opponent at dr.med. Eystein S. Husebye's disputation on the work: "Stimulus-secretion coupling in chromaffin cells of the bovine adrenal medulla. With special reference to the role of phospholipid metabolism". University of Bergen, Norway, 1990.

11. Opponent at dr.scient. Hooshang Lahooti's disputation of the work: "The estradiol receptor and the 90 kDa heat shock protein. Phosphorylation of the receptor and the heat shock protein, and studies on regulation of the estradiol receptor mRNA". University of Bergen, Norway, 1991.

12. Leading the disputation of Hilde Nebb Sørensen on the work: "Hormonal modulations of fatty acid stimulated peroxisomal ß-oxidation in cultured liver cells." University of Oslo, Norway, 1993.

13. Opponent at dr.odont. Janicke Liaaen Jensen's disputation on the work: "Human saliva: Biochemical and physiological aspects of some components", Faculty of Density, University of Oslo, Norway, 1994.

Member of advisory international/national committies for evaluation of professor positions.

1. Professor Ingrid U. Richardson, Harvard University, Boston, USA. 1974.

2. Professor Thomas F.J. Martin, University of Wisconsin, USA. 1984.

3. Professor Margaret A. Brostrøm, University of Medicine and Dentistry of New Jersey, USA. 1984.

4. Position as full Professor at the Institute of Physiolology, University of Gothenburg, Sweden. 1987.

5. Professor I at the Department of Clinical Chemistry, University of Tromsø, Norway, 1987.

6. Participation in an international board created by Sandoz, Basel, Switzerland, for nominating a candidate for the Sandoz International Endocrinological Prize in 1988.

7. Professor in Veterinary medicine, Norwegian Veterinary University, Oslo, Norway, 1989.

8. Position as Professor in Physiological Chemistry, University of Kuopio, Finland. 1989.

9. Position as Professor I in Endocrinology at the University of Gothenburg, Sweden, 1991.

10. Position as Associate Professor I in Endocrinology, University of Gothenburg, Sweden, 1993.

11. Position as Associate Professor I in Biochemistry, University of Bergen, Norway, 1993.

12. Appointed member of committy to evaluate chair Professorship at Karolinska Institution, Dept. of Endocrinology, Sweden (I had to decline because of sabattical year).

Honorary Societies

Member of the Norwegian National Academy of Science and Letters

Professional memberships

Norwegian Society of Biochemistry
Norwegian Society of Physiology
Norwegian Society of Endocrinology
Endocrine Society (USA)
Society for Calcified tissue (USA)
Society for Bone and Mineral Metabolism (USA)

Medical clinical specialities

1. Clinical Physiology and Chemistry including Nuclear Medicine
2. Work Medicine

Medical Faculty Responsibilities

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Reesearch Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's councel for evaluation of postgraduate applications from 1989-1993.

Research Council Responsibilities

1. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
2. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
3. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
4. Member of the International Scientific Board of Novo-Nordisk Research Committee.
5. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway.

Patents

General information

Two U.S. patents, U.S. Patent No. 5.010 010 and No. 5.420.242 are held with

international extensions in Europe, Japan, Canada, and Australia. In addition, three Divitional Applications are submitted to the U.S. Patent Office and elsewhere.

All of Gautvik et al.'s patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms.

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS
GIVEN IN HISTORICAL ORDER:

I.   THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO
      PLASMA KININS AND KALLIKREINS

II.   MOLECULAR ENDOCRINOLOGICAL RESEARCH

      A.   REGULATION OF HORMONE SECRETION AND SYNTHESIS

      B.   RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE
            HORMONE RECEPTORS

      C.   STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A
            NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

III. ENDOCRINOLOGICAL RESEARCH RELATED TO HYPERFUNCTION OF
      THE PARATHYROID GLAND AND RESEARCH IN RELATION TO
      MEDULLARY CARCINOMA OF THE THYROID GLAND

      A.   CLONING AND EXPRESSION OF PARATHYROID HORMONE AND
            RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS
            AND TRANSGENIC ANIMALS

      B.   PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT
            HUMORAL HYPERCALCAEMIA

      C.   ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR
            THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION
            HYBRIDIZATION

      D.   ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR
            PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

IV.   MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

      A.   STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH

      B.   ISOLATION, PURIFICATION, AND CHARACTERIZATION OF S
            ALMON PROLACTIN AND GROWTH HORMONE

I.   **THE BIOCHEMICAL AND PHYSIOLOGICAL STUDIES RELATED TO PLASMA KININS AND KALLIKREINS**

Plasma kinins are biologically very active polypeptides that are distributed throughout the body and become accepted to be of importance for regulation of blood flow in certain organs. Kinin forming enzymes (kallikreins) and kinin inactivating enzymes (peptidases, "converting enzyme") have received new attention the last years due to their possible involvement in hypertension. My thesis from April 1970 dealt with certain physiological/biochemical aspects in relation to blood flow regulation including purification of substrates for kallikreins and their characterization in vitro. These components were used for studying their physiological interaction during perfusions of the salivary gland activated via nerve stimulation. By using purified substrates and their enzymes, a direct functional involvement of plasma kinins in functional vasodilatation could be demonstrated in vivo for the first time. Its title was "Studies on vasodilator mechanisms in the submandibular salivary gland in cats" (O.A.: 7).
Relevant references: R/C, 3,6,7,8,11,12,13; O.A, 1-6,8,9,11,16,19,23, 39,43,50, 55,56.

II.   **MOLECULAR ENDOCRINOLOGICAL RESEARCH**

A.   **REGULATION OF HORMONE SECRETION AND SYNTHESIS**

A major part of my research engagement has been carried out using functional cell cultures and transplantable tumors from highly differentiated cells that are able to perform organ specific functions. The following areas have been actively persued since 1971:

i.   **The biological effects and mechanism of actions of the hypothalamic hormones thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide in prolactin and growth hormone producing rat pituitary cells.**

These hypothalamic hormones are of central importance in regulation of release of prolactin and growth hormone from the anterior pituitary gland. The results which we have obtained with the cultured rat pituitary cells, have all been confirmed in more physiological endocrine model systems and thus appear to be valid for interpretation of how these regulatory hormones influence the functions of the anterior pituitary gland. I have carried out characterization of receptor binding of thyroliberin and been a senior researcher to originate research regarding receptor characterization for dopamine, somatostatin and vasointestinal polypeptide. In addition, I have steadily persued studies to elucidate and delineate the mechanisms of action for these peptide hormones. We have characterized the second messengers systems involving cyclic nucleotides and calcium, as well as described pathways of phospholipase C activation with formation of inositol triphosphates and

diacylglycerol. I have been one of the first in this research area to show the involvement of cyclic nucleotides and calcium in the action of thyroliberin, dopamine, somatostatin and vasoactive intestinal polypeptide. How these second messengers were generated and their interaction, were first described in an invited review article for the Benzon Symposium, Copenhagen, 1988. (K.M. Gautvik et al., Regulation of prolactin secretion and synthesis by peptide hormones in cultured rat pituitary cells, Alfred Benzon Symposium 25, Copenhagen, 1988) and as an invited lecture at the 2nd European Congress in Ljubljana, 1990.

The ongoing research has concentrated on the involvement of GTP binding proteins in the receptor coupling of these hormonal signals as well as the characterization of the receptor itself. Thus we have identified and studied the functional coupling between these receptors and the G protein subunits in pituitary cells. Furthermore, these studies are now completed with a description of the adenylyl cyclase subclasses in the same cell-types and their engagement by the different hormone-receptor G protein subunits. These studies have involved measurements of specific mRNAs, the corresponding proteins and their regulation by the hypothalamic hormones as well as antisense RNA experiments testing the direct physiological involvement of G_{α_s} protein in the action mechanism of e.g. thyreoliberin.

ii)   The biological effects and mechanisms of action of steroid hormones (oestradiol, progesterone, testosterone, cortisone and vitamin D,) examined in prolactin and growth hormone producing cells in culture.

Through the years 1973-1983, I was engaged in studying the effects of the above mentioned steroid hormones and characterization of their distinct receptors in prolactin and growth hormone producing cells in culture. We showed e.g. for the first time the existence of testosterone and vitamin D_3 receptors in adenopituitary cells. The biological effects of these hormones and how they regulate prolactin and growth hormone synthesis, were also examined in detail. How the steroid and polypeptide hormones regulated hormone receptor levels was studied during different conditions with the aim to understand their physiological interplay.

iii)   Regulation of rat prolactin and growth hormone gene expression in functional pituitary cells.

Many of the hormones which affects prolactin and growth hormone secretion are also able to change the rate of synthesis for these hormones. In the same decade, we developed immunopresipitation methods for the radioactively labelled hormones, and improved the sensitivity of hormone measurements to the level of single cells. This was shown using capillary tube gel electrophoresis of immunoprecipitated hormones and the results confirmed by concommitant immunocytochemistry. By using a combination of protein analysis and RNA blotting methods, we could show that prolactin synthesis was stimulated mostly by thyroliberin and oestrodiol while an inhibitory effect was found by dopamine and cortisone. The most efficient inducer of growth hormone synthesis was cortisone, and its

synthesis was also inhibited by dopamine.
Relevant references: O.A.: 12-14,22,24,25,27,31-34,36-38,42,44,48,49,51,57-59,62,66,
67,69,71-73,76,77,80,81,83-85,87,91,92,96-105,107,109,110,112,114,115,122,132,
133,134; R/C: 17,19,21,22,23,26,28-30,34.

B.   RECEPTOR FUNCTION AND CLONING OF NEUROENDOCRINE
       HORMONE RECEPTORS

In this respect, we have isolated and cloned cDNA for the receptor for thyroliberin in rats
and in humans, the latter result as the first original description in the litterature. Prior to
this work we characterized and visualized this receptor both by photo affinity labelling
using the radioactive hormone as well as using a polyclonal antiserum made by us and
raised against cell surface ideotypes. We cloned the rat and human receptor after making a
mRNA based PCR product and a cDNA which was then used as probe for screening
libriaries.

The human thyroliberin receptor, shows several interesting features when the aminoacid
sequences are compared in rat and human. The differences have probably direct bearing on
the functional activity of the receptors in relation to G protein coupling pattern as well as
signal effector activation, which we in the rat have described in detail.

Relevant reference: O.A. 115,130,157.

C.   STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL
       SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The hypothalamus consists of discrete nuclei which paly a vital role in several biological
functions that are essential to mammals and related to different homeostatic mechanisms,
reproduction, behavior, emotion and responses to various stress. The hypothalamic nuclei
in part, integrate many autonomic regulatory systems whose final path is expressed by
neuroendocrine cells. Thus, they represent "high command centers" within the endocrine
system and enable the central nervous system to initiate, adjust and balance intricate and
complex endocrinological reflexes. This part of hypothalamic function is exerted by
synthesis and release into the pituitary portal system of a number of substances, mainly of
peptide and amine nature. These substances control the anterior pituitary gland function.
In addition, hypothalamus is the site of production of two hormones, oxytocin and
vasopressin, which are transported by axon flow into the posterior pituitary from which
they are released into the general circulation affecting salt/water balance and being of

importance for parturition and breast feeding. Furthermore, the hypothalamus is a center for social and sexual biological behaviors and mediates feeding and drinking habits. The importance of hypothalamic functions in the field of calorie balance may be exemplified by the recent discovery by Zhang et al. (Nature, 372, 1994) who describe the first cloning of the so called mouse "obese" gene and its human analog, where the receptor for this hormone by all criteria has to be localized in a hypothalamic nucleus. Since the first objective of this research has been obtained, namely to generate a subtracted hypothalamic specific cDNA library of high quality, we will expect to find this receptor among the 10^5 independent clones present in the library, as well as clones of mRNA encoding a large number of other novel proteins.

The subtraction library shows inserts with sizes estimated on agarose gel electrophoresis between 0.4 and 1.2 kb (mean > 0.7 kb), a result which was very satisfactory.

The quality of the library is further assessed by the extent to which clones of certain mRNAs known to be present in hypothalamus had been amplified during subtraction, and to what extent clones of mRNAs which are ubiquitously present in the central nervous system had been removed. Vasopressin which is exclusively present in hypothalamus is enriched 20-30 times in the subtracted library compared to the hypothalamus cDNA library, and the commonly present NSE and cyclophilin is completely removed after subtraction. 215 clones from the subtracted library have been picked into grids and hybridized with probes prepared by PCR amplification of the inserts from the driver, target and subtracted libraries. Approximately 1/4 of the clones give substantially greater signals with the subtracted target probe than the unsubtracted target probes and faint or undetectable signals with the driver probe. If validated, these figures suggest that roughly 1 % of the hypothalamus mRNA mass is enriched in that structure (corresponding to an estimated 300 different gene species, given that 30,000 species are expressed in the brain).

Conclusion: The results so far from our learning and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method are summarized. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. One article on the general aspects of hypothalamic enriched/specific mRNAs is in preparation. Another article is describing a novel somatostatin-like peptide, called cortistatin. This article is in press 1996 in Nature.

III.   ENDOCRINOLOGICAL RESEARCH RELATED TO
        HYPERFUNCTION OF THE PARATHYROID GLAND AND
        RESEARCH IN RELATION TO MEDULLARY CARCINOMA OF
        THE THYROID GLAND

This research started out in the early 70'ies as a result of my development of two radio-immunoassays for parathyroid hormone and calcitonin, methods which at that time did not exist in Norway. Since 1973, I have thus carried out clinical laboratory diagnostic activity

for the whole country, and also received samples from other Scandinavian countries as well as England. In different collaborative studies, we used these assays in basal and clinical endocrinological research related to how these hormones were regulated by calcium both in vitro and in vivo. In addition, biochemical work was carried out in order to characterize different intracellular hormone-forms retrieved from tumor cells producing these peptides.

The hyperfunction of the parathyroid glands occurs in relation to development of adenomas and/or hyperplasia. The cause(s) of primary hyperparathyroidism is (are) unknown, while secondary hyperparathyroidism occurs as a result of chronically lowered serum Ca^{2+} (e.g. in chronic renal failure). The question about how low concentration of serum Ca^{2+} may induce not only increased hormone secretion and synthesis, but also trigger DNA replication and cell proliferation, is intriguing, but still unknown. We have studied patients with secondary hyperparathyroidism during various experimental conditions to address these questions. From human adenomas we isolated poly(A)$^+$ RNA for cloning of parathyroid hormone (PTH) in 1983.

Medullary carcinoma of the thyroid gland (MCT) occurs as an inherited and spontaneous malignant disease. As the first to introduce diagnostic tool to discover this calcitonin producing tumor in Norway, we have mapped the extent of the disease in our country.

Relevant references: O.A.: 28-30,40,41,52-54,61,65,68,70,74,78,79,82,86, 90,94,108, 117; R/C: 5,15,16.

A.   CLONING AND EXPRESSION OF PARATHYROID HORMONE AND RELATED PEPTIDES IN MICROORGANISMS, MAMMILIAN CELLS AND TRANSGENIC ANIMALS

This project started in 1983 and developed into a major research engagement where we have succeeded as the first in the world, to clone and express this hormone in 100 mg quantities both as a product in E.coli as well as in Saccharomyces cerevisia. We have also successfully transfected mammalian cell cultures, insect cells and transgenic animals, silkworm larvae and mice, and obtained expression of this hormone. We have also expressed mutated forms in yeast and studied the intact hormone and fragments in insect cells and Bombyx mori larvae with the aim to understand cellular processing, traficking and secretion.

In the first part of the work we cloned the cDNA for human parathyroid hormone using conventional cloning techniques and expressed the peptide as met-gly product in E.coli. This peptide analogue was not biological active. In a search for peptides with antagonistic action, we found that gly PTH (1-84) was an interesting form having binding properties and inhibited the agonist by 40 per cent at $10^{-9}M$. In clinical medicine an effective PTH antagonist will be of potential use both as a diagnosticum and in treatment of hypercalcaemia.

For expression in S.cerevisiae recloning of the cDNA was carried out using a fusion construction with the prepro region of the yeast mating factor α gene. We were able to obtain an effective and correct N-terminal processing and isolated human PTH as a secretory product in a yield (up to 10 mg/l). We also developed a down-stream technology for purifying the hormone which was shown to be identical to the authenthic peptide hormone by a variety of chemical, biochemical and biological test systems.

In order to obtain a higher yield of human parathyroid hormone, we in vitro mutagenized a proteolytic cleavage site internal to the peptide, and obtained a full-length agonist (84 amino acids) which after purification was shown to have the same biological activity as the authentic hormone.

A part of this work has been concentrated on to find optimal signal sequences both for expression in E.coli as well as in yeast, where we by using different amino acid substitution in new gene constructs, have developed an effective test system for looking at N-terminal processing. This is obtained by making a fusion gene between the N-terminal region of the PTH gene and the protein A gene which in E.coli is transcribed using the protein A promoter and transcription stop signals.

We have also made constructs for use in mammalian cells where we employ the Whey Acidic Protein (WAP) promoter region in order to express PTH in mammalian cells of mouse origin. We have recently in addition generated transgenic mice who express PTH as a secretory product in milk.
Relevant references: O.A.: 124,128,129,133,139,140,144,145,161,162,163,166, 167,168; R/C: 31,32,35.

B. PARATHYROID HORMONE RELATED PEPTIDE AND MALIGNENT HUMORAL HYPERCALCAEMIA

Parathyroid hormone related peptide has been isolated as the causative agent during conditions of malignant humoral hypercalcaemia. This condition occurs in the presence of several malignent diseases such as cancers, carcinomas as well as myelomatosis where the tumour cells are able to produce a parathyroid hormone related peptide. We have received cDNA clones and recloned it in yeast for production of the protein to be used in receptor binding- and activation-studies. We are also studying the expression of this gene in animal and human tumour cells, with the purpose to learn gene specific splicing.
Relevant references: O.A.: 151; 160, Jemtland et al., Rian et al. submitted; R/C: 36.

C. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE OSTEOSARCOMA PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described in

Chapter II,C, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual cones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumortype.

D. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of sentral importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular substraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is in its initial phases.

IV. MOLECULAR ENDOCRINOLOGY STUDIES IN FISH

A. STUDIES OF GENE EXPRESSION IN TRANSGENIC FISH

The endocrinological aspects as it relates to regulation of growth and development of fertility, are as important in fish as in mammalian species. In addition, fish is an i nteresting model system also for studies within embryology, differentiation, and gene regulation. As in mammalian species, growth hormone will regulate growth and prolactin will be of importance for normal fertility and adaptation to salt/fresh water conditions. Also, in this research area, we have worked partly from the protein side and partly from the DNA side. We have as first reports described isolation and purification of prolactin and growth hormone from Atlantic salmon, and developed sensitive radioimmunoassays in order to follow the hormones in fish as a function of age and also during different experimental conditions.

The initial DNA work for production of transgenic fish is published and may be summarized briefly:

We first developed a new microinjection technique where small amount of foreign DNA was injected into fertilized fish eggs and the survival rate was more than 90%.

As the first model gene we used the human growth hormone gene (kindly given from Professor R. Palmiter, USA) where the promoter for the metallothionin gene ensured expression in eukaryotic organisms. From this gene construction, we made cDNA probes which specifically hybridized to growth hormone DNA and mRNA.

The microinjected DNA for human growth hormone gene incorporated in the embryo's chromosomal DNA was isolated and demonstrated by Southern blot analysis. It was incorporated in the fish genome already after 7 days.

We also showed that the human growth hormone gene was active based on the occurrence of specific mRNA for human growth hormone and production of growth hormone by the fish embryo and secretion to the medium. This achievement shows that it is possible to develop a rather unique model to study gene expression both under embryonic development and in the adult fish (R/C 44; O.A. 119, Skibeli submitted 1996).

In order to measure expression of Atlantic salmon growth hormone, we have isolated and purified this hormone as well as prolactin from the same species. (O.A. 118, 121, 135, 138).

B. ISOLATION, PURIFICATION AND CHARACTERIZATION OF SALMON PROLACTIN AND GROWTH HORMONE

At the time we started out this project, a preliminary sequence of the corresponding hormones in the Pacific salmon was known. During this project we were however, able to purify and characterize both these hormones from the Atlantic salmon and were the first to give the amino acid sequence data on both these hormones. In addition, we used our own made prolactin antisera for studying the possible function and involvement of prolactin in sex maturation of Atlantic salmon. Growth hormone was further characterized by detailed chemical and biochemical analysis including phosphorylation and glycylation patterns, development of antisera against fragments of the hormone, and analysing the immunoreactivity of growth hormone from different salmon species. These reports describes for the first time GH species as two gene products in Salmon fish, and they are both glycoproteins, and one also phosphoglycoprotein.

Relevant references: R/C 44; O.A. 118, 119, 135, Skibeli submitted 1996).

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 4 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I.   PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE
      RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

i)   Recombinant parathyroid hormone for structure activity studies in relation to bone
      cell activation.
ii)   Study intracellular processing and trafficking of these hormones and to compare
      signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant
parathyroid hormone in mg quantities. For this work we developed gene constructs, vector
modifications, fermentation technological improvements as well as complete methods for
down-stream technology. The final product is PTH identical and more than 99% pure and
has shown full chemical, biochemical and biological identity with the intact hormone.
These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to
express secreted human parathyroid hormone in mammalian cells as well as a secretory
milk product in transgenic mice. In addition, we have been the first to develop full-length
PTH polypeptides with agonist and antagonist functions.

1.   Høgset A, Blingsmo OR, Gautvik VT, Sæther O, Jacobsen PB, Gordeladze JO,
Alestrøm P, Gautvik KM. Expression of human parathyroid hormone in Escherichia coli.
BBRC, 166:50-60, 1990.
2.   Gabrielsen OS, Reppe S, Sletten K, Øyen TB, Sæther O, Høgset A, Blingsmo OR,
Gautvik VT, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and secretion of
human parathyroid hormone in Saccharomyces cerevisiae. Gene, 90(2): 255-262, 1990.
3.   Høgset A, Blingsmo OR, Sæther O, Gautvik VT, Holmgren E, Josephson S,
Gabrielsen OS, Gordeladze JO, Alestrøm P, Gautvik KM. Expression and characterization
of a recombinant human parathyroid hormone secreted by E.coli employing the staphylo-
coccal protein A promoter and signal sequence. J. Biol. Chem., 265: 7338-7344, 1990.

In this regard we have received acceptance for an international patent on gene construc-
tions, plasmids, the process and the down-stream technology.

In the further work we have by using in vitro mutagenesis, created full length parathyroid
hormone agonist which has shown to be protease resistant and have interesting biological
actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

4. Reppe, S., Olstad, O.K., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Gabrielsen, O.S., Øyen, T.B., Gordeladze, J.O., Haflan, A.K., Tubb, R., Morrison, N., Tashjian, A.J. Jr., Alestrøm, P., Gautvik. K.M. Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product. ECB, 5th European Congress on Biotechnology, Copenhagen July 8-14, 1990. (Invited).

5. Reppe, S., Gabrielsen, O.S., Olstad, O.K., Morrison, N., Sæther, O., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Haflan, A.K., Voelkel, E.F., Øyen, T.B., Tashjian Jr., and Gautvik. K.M. Production of recombinant human parathyroid hormone in yeast: Synthesis, purification, and biological characterization of a Lys-26–Gln site directed mutant. J. Biol. Chem., 266: 14198-14201, 1991.

6. Olstad, O.K., Reppe, S., Gabrielsen, O.S., Hartmanis, M., Blingsmo, O.R., Gautvik, V.T., Haflan, A.K., Christensen, T.B., Øyen, T.B., Gautvik. K.M. Isolation and characterization of two biologically active O-glycosylated forms of human parathyroid hormone produced in Saccharomyces cerevisiae. Identification of New Motif for O-glycosylation. Eur. J. Biochem., 205:311-319, 1992.

7. Forsberg, G., Brobjer, M., Holmgren, E., Bergdahl, K., Persson, P., Gautvik. K.M., and Hartmanis, M. Thrombin and H64A subtilisin cleavage of fusion proteins for preparation of human recombinant parathyroid hormone. J. Protein Chem., 1991, 10(5): 517-526.

8. Kareem, B.N., Rokkoknes, E., Høgset, A., Holmgren, E., Gautvik. K.M. A method for the evaluation of the efficiency of signal sequences for secretion and correct N-terminal processing of human parathyroid hormone produced in Escherichia coli. Analytical Biochemistry, 1992, 204: 26-33.

In our ongoing studies regarding the mapping of functional domains in human parathyroid hormone in comparison with the parathyroid hormone-like protein (PTHrP) we have expressed the human forms successfully in Saccharomyces cerevisiae and have also cDNA clones for their receptor as well as permanently transfected mammalian cells which express the receptor on the surface. By having access to PTH and PTH analogues as well as PTHrP, we are in a good position to map out the binding affinities of different hormonal forms as well as their coupling to different cellular signal systems.

Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding K_D which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.

9. Rian, E., Jemtland, R., Olstad, O.K., Gordeladze, J.O., Gautvik. K.M. Expression of biologically active human parathyroid hormone-related protein (1-141) in Saccharomyces cerevisiae. Eur. J. Biochem. 213: 641-648, 1993)

10. Rian, E., Jemtland, R., Olstad, O.Kr., Endresen, M.J., Grasser, W.A., Thiede, M.A., Henriksen, T., Bucht, E., Gautvik. K.M. Parathyroid hormone-related protein is produced by cultured endothelial cells; A possible role in angiogenesis. Biochem. Biophys. Res.

Commun., 198(2): 740-747, 1994.

11. Kareem, N.B., Rokkones, E., Høgset, A., Holmgren, E., Gautvik, K.M. Translocation and processing of various human parathyroid peptides in E.coli are differentially affected by protein A signal sequence mutation. Eur. J. Biochem, 220: 893-900, 1994.

12. Rokkones, E., Kareem, B.N., Olstad, O.K., Høgset, A., Schenstrøm, K., Hansson, L., Gautvik, K.M. Expression of human parathyroid hormone in mammalian cells, Escherichia coli and Saccharomyces cerevisiae. J. Biotechnol., 33: 293-306, 1994.

13. Olstad, O.K., Morrison, N.E., Jemtland, R., Jüppner, H., Segre, G.V., Gautvik, K.M. Differences in binding affinities of human PTH(1-84) do not alter biological potency: A comparison between chemically synthesized hormone and recombinant forms. Peptides, 15: 1261-1265, 1994.

14. Rokkones, E., Fromm, S.H., Kareem, N.B., Olstad, O.K., Høgset, A., Klungeland, H., Iversen, J., Bjøro, K., Gautvik, K.M. Human parathyroid hormone as a secretory peptide in milk of transgenic mice. Journal of Cellular Biochemistry, 59, 168-176 1995.

15. Olstad, O.K., Jemtland, R., Loseth, O.P., Bringhurst, F.R., Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone partial agonist with antagonistic protperties: Gly-hPTH(-1-+84). Peptides, vol. 16, 1031-1037, 1995.

16. Mathavan, S., Gautvik, V.T., Rokkones, c., Olstad, O.K., Kareem, B.N., Maeda, S., Gautvik, K.M. High level production of human parathyroid hormone in Bombyx mori larvae and BmN cells using recombinant baculovirus. Gene, vol. 167, 33-39, 1995.

17. Rian, E., Jemtland, R., Løseth, O.P., Gautvik, K.M. Characterization of mRNA 3-end formation in Saccharomyces cerevisiae detected by the 3'untranslated regin of human PTHrP(1-141) transcripts. Submitted.

18. Olstad O.K., Jemtland, R., Bringhurst, F.R., Gautvik, K.M. Expression and characterization of a partial recombinant human parathyroid hormone antagonist: Gly-hPTH(-1-+84). Manuscript, 1996.

The various recombinant PTH and PTHrP forms will be studied in their interaction with natural receptors of bone cells in culture and also in relation to recombinant receptor permanently transfected in mammalian cells. The aim of this is to understand in more detail the structure activity relationship between the different hormonal forms and their ability to activate different cellular signalling systems. The ultimate goal will be to try to understand how the osteoblast is activated by parathyroid hormone in the bone remocelling process of importance for the elucidation of the causes and patogenesis of osteoporosis.

II.   NEUROENDOCRINE RECEPTORS AND THEIR FUNCTION

During our cDNA cloning of G-coupled receptors in rat pituitary cells and in human CNS, we have identified four potential candidates for G protein coupled receptors distinct from the TRH clone in a human phage library. We were able to isolate and characterize a functional human TRH receptor and to present these results as the first original international report. In addition to engaging in characterization of the other receptors, we are at present working on the organization and functional aspects of the gene for the human TRH receptor.

The ongoing and future research will concentrate on:

1. To map deleted receptor cDNA clones for functional activity using the Xenopus laevis oocyte system as a hormone (TRH) specific bioassay.
2. Generation of transfected cells to map out the hormone-binding receptor region as well as dissect which part of the hormone receptor couples to the two previously characterized G proteins which mediate signal system activation conveying its physiological actions, namely the $G\alpha s$ coupling to the adenylyl cyclase system and the Gq/11 coupling to the phospholipase C.
3. Making hybrid receptors between the thyroliberin and the PTHrP/PTH receptor in order to analyze further the importance of the different receptor domain for conveying signal transduction.
4. Analyse the 5'-end of a newly isolated genomic clone for the human receptor.

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Two manuscripts in preparation.

III. STUDIES OF THE INTERACTION BETWEEN FATTY ACIDS AND DEXAMETHASOME AND INSULIN REGARDING REGULATION OF PEROXISOMAL ß-OXIDATION ENZYMES. POSSIBLE INVOLVEMENT OF THE PEROXISOMAL PROLIFERATOR ACTIVATED RECEPTOR (PPAR) GENE AND ITS REGULATION

On a collaborative basis within my institute, I have since 1991 engaged in research regarding hormonal control of peroxisomal ß-oxidation enzymes triggered by a surprising finding that fatty acids and dexamethasome have strong synergistic actions in regulation of the transcription of these three enzyme genes. This positive cooperativity was completely blocked by insulin in cultured liver cells and also in intact rats. The studies were carried out on the RNA, protein and enzyme activity levels and opened a new side of this already very much studied area of fatty acid ß oxidation. The ongoing research in this area will continue as a collaborative work between professor Jan-Åke Gustafsson's group at Huddinge Hospital and Institute for Medical Biochemistry, where we will concentrate on delineating the possible regulatory elements located in a genomic clone of the PPAR from rat.
Relevant O.A.: 120,125,127,131,147,150.

IV THE USE OF A NOVEL SENSITIVE MOLECULAR SUBTRACTION HYBRIDIZATION METHOD FOR STUDYING DIFFERENTIALLY EXPRESSED mRNAs

Studies of hypothalamic specific mRNAs obtained by subtraction hybridization procedure

Isolation and characterization of mRNAs specific for the osteosarcoma phenotype obtained by subtraction hybridization

Isolation and characterization of mRNAs psecific for parathyroid hormone gene activation in bone cells

Conclusion

This ongoing work has very successfully been able to isolate unique hypothalamic specific mRNAs among those also a novel somatostatin-like peptide. In addition, this subtracted library will probably contain the long sought for receptors which fatty acid or their metabolites are acting on, in order to regulate calorie intake and consumption. This bridges then the research going on in Chapter III and its work. The unique subtracted hypothalamic library can be exemplified with the finding that we also have isolated a novel calcium calmodulin kinase whose distribution is unique in CNS and also a transmembrane protein of secretory vesicles which has never previously been cloned in mammalian species, but has its homology in the electric organ of the electric eel. The characterization and studies of full-length cDNA clones from these mRNAs are given highest priority.

Already the differential display of mRNAs present in human osteosarcoma cells and absent in normal bone cells is very promissing and certainly leads to encouraging considerations regarding the possibility to obtain osteoporosis specific genes as defined by PTH specific mRNAs in normal osteoblasts.

P U B L I C A T I O N   L I S T

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of   :
    Gautvik et al..   :
                          :   Group Art Unit: 1812

Serial No. 08/340,664   :
                          :   Examiner: L. Spector

Filed: November 16, 1994   :
                          :

For: PRODUCTION OF HUMAN   :
    PARATHYROID HORMONE FROM   :
    MICROORGANISMS   :
_____X

Assistant Commissioner for Patents
Washington, D. C. 20231

DECLARATION OF JOHN E. MAGGIO, Ph.D. PURSUANT TO 37 C.F.R. § 1.132

Sir:

     I, John E. Maggio, declare as follows:

     1.   I am a citizen of the United States of America residing at 480 Washington Street, Brookline, Mass. 02146.

     2.   I am an Associate Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School, Boston, Massachusetts. I have been a professor at Harvard Medical School since 1985.

     3.   My formal education includes Bachelors, Masters and Doctoral degrees, in the fields of chemistry and organic chemistry, all from Harvard University. I have also had extensive post-doctoral training at the University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge University, Cambridge, England, and at the Neuropsychopharmacology Research Unit at Yale University Medical School in New Haven. Both post-doctoral positions involved protein and peptide chemistry and purification thereof. My current curriculum vitae, including a list of my publications is attached as Exhibit 1.

4. My past and present work centers around synthesis, purification and characterization of biologically active peptides including tachykinins, magainins and amyloid peptides. As part of my work and since at least the late 1970s, I have used various forms of chromatography and electrophoresis for the purification of peptides and for their characterization. I am fully familiar with these techniques, and the state of their development throughout the 1980s and 1990s. I am also fully familiar with the past and present capabilities and limitations of such techniques. Representative of my work with peptide separation and characterization is an article attached as Exhibit 2 entitled "Mapping Peptide-binding Domains of the Substance P(NK-1) Receptor from P388D$_1$ Cells with Photolabile Agonists", *J. Biological Chemistry.* 270, (1995), 1213-1220.

5. My knowledge of chromatography, electrophoresis, and other techniques used commonly in protein chemistry stems from my repeated use of those techniques throughout my career. I have supervised students and other scientists using these techniques and have taught the techniques, both in the classroom and in the laboratory. Therefore, I am comfortable judging the ordinary level of skill that a person in this art would possess in terms of the theoretical and the bench aspects of these techniques.

6. In preparing this declaration I reviewed, among other things, the following materials: a copy of the specification of U.S. Serial No. 08/340,664, filed November 16, 1994, (the "'664 application"); a copy of the Official Action dated September 8, 1995 issued by the patent examiner, Dr. Spector; copies of each of the references identified in the

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Official Action; and a copy of the Declaration of Kaare M. Gautvik, M.D. Pursuant to 37 C.F.R. § 1.132 as well as the documents and photographs attached thereto.

7.   I understand from my review of the Official Action, the United States Patent and Trademark Office has refused to grant the '664 application in view of the disclosures of four references: *Brewer et al., Fairwell et al., Kimura et al.* and/or *Kumagaye et al.*

8.   I have reviewed the four references cited by the Patent Office and I do not agree with the Patent Office's conclusions regarding their teachings or disclosures. In my opinion, none of the references describe or suggest a method of obtaining a substantially pure, intact, hPTH peptide. None of the references provides a basis for concluding that a substantially pure hPTH product was actually produced. Further, nothing in the references describes an hPTH peptide having biological activity substantially equivalent to naturally occurring hPTH. I believe that a biochemist, organic chemist or analytical chemist having an ordinary level of skill in this technology would not be unable to draw any conclusion with regard to the purity of hPTH produced in accordance with the cited references. If anything, given the errors appearing in those references and the known shortcomings of the techniques described in the references, *e.g.* solid phase chemical synthesis, those of ordinary skill in the art would probably assume that the resulting hPTH material was impure.

9.   *Brewer et al.* relate to an isolation from tissue, not a recombinant material. *Brewer et al.* contain three errors at positions 22, 28 and 30 of the synthesized peptide compared to the wild-type peptide. This is illustrated

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in Fig. 1 of *Brewer et al.* Accordingly, *Brewer et al.* do not teach the production of an *intact* hPTH peptide. Moreover, two later publications cited by Dr. Spector in the Official Action, namely *Kimura et al.* and *Kumagaye et al.,* show that the purification protocols discussed in *Brewer et al.* result in impure materials. For example, Fig. 2, on page 496 of *Kimura et al.* is an HPLC profile of crude product obtained after use of a separation protocol analogous to that disclosed in *Brewer et al.;* namely, the use of a combination of gel filtration and ion exchange chromatography. Impurities are plainly evident. Therefore, a conclusion of homogeneity based on *Brewer et al.* is unjustified. Further, *Kimura et al.* describe a purification sequence of CM-cellulose column chromatography followed by gel filtration on Sephadex G-50, followed in turn by the use of reverse phase-high pressure liquid chromatography ("RP-HPLC"). *Kimura et al.* added the RP-HPLC step in recognition of the need to obtain better purity than *Brewer et al.* obtained. This fact alone, in my opinion, eliminates any plausible basis for concluding that the protein resulting from the methods described in *Brewer et al.* was essentially pure.

10. Many of the criticisms of *Brewer et al.* apply to *Fairwell et al.* For example, *Fairwell et al.* produced a peptide having an Asp at position 76. Native hPTH has an Asn in that position. *Fairwell et al.* also used a separation protocol combining the use of gel filtration and ion exchange chromatography. As previously mentioned, that protocol was re-run by *Kimura et al.* and the results, as illustrated in the chromatogram in Fig. 2 thereof, show significant impurities. Finally, as *Kimura et al.* used RP-HPLC rather than relying

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merely on the separation protocol described in *Fairwell et al.*, it is clear that subsequent investigators believed that the separation protocols of *Fairwell et al.* were inadequate.

11. *Kimura et al.* did not produce an essentially pure hPTH. As *Kumagaye et al.* clearly explain, "[t]oday, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products." (Emphasis added) *Kumagaye et al.* at page 330. This is especially significant because *Kumagaye et al.* is the same group of researchers as *Kimura et al.*

12. *Kumagaye et al.* disclose a method of separating two different forms of hPTH from a mixture thereof by using cation exchange-HPLC. This is not a particularly surprising result as the two forms of hPTH disclosed have a full charge difference between them, a situation ideal for the use of cation exchange-HPLC. Nonetheless, one of ordinary skill in the art would not conclude that the resulting hPTH in accordance with *Kumagaye et al.* was essentially pure. One could conclude that the resulting material was pure of the one specific impurity, i.e. the specific point mutated form disclosed. However, there is no basis for concluding further. If anything, as explained herein, there is every reason to believe that impurities are present.

13. *Kumagaye et al.* describe a solid phase peptide synthesis protocol which was common at the time. That synthesis, as explained in the *Kimura et al.* article, involved the use of BOC protected amino acids and traditional BOC chemistry. Using BOC chemistry, each successive amino acid is

added to the N-terminus of a growing chain by first removing the blocking group in acid, then neutralizing prior to coupling, followed by coupling the next BOC amino acid in sequence using, for example, dicyclohexylcarbodiimide ("DCC").

14. This technology suffers from a number of well known shortcomings and, in fact, has largely been replaced. One of the better known and most common problems with solid phase BOC chemistry, particularly for longer peptides such as hPTH, is racemization. As explained in *Bodanszky,* "Peptide Chemistry; A Practical Textbook" at page 120, the problem of racemization using DCC coupling and BOC chemistry is well documented. *See* Exhibit 3. This chapter, as well as the others attached as Exhibit 3, demonstrate the prevalence of racemization and concerns over this phenomenon during solid phase protein synthesis.

15. Some of the other well known impurities generated by solid phase synthesis are described in the *Fairwell et al.* article cited by Dr. Spector at page 2691. These impurities include, among other things, deletion peptides, omission peptides and prematurely terminated peptides. For example, during solid phase synthesis it is possible for coupling to be either duplicative or incomplete, thereby providing a peptide having an additional amino acid or an omission from the normal sequence. There may be one or more additions and/or deletions in any given peptide. These additions and/or deletions can occur almost anywhere along the chain. Premature termination of the chain length is also common. This may occur for a host of reasons such as, peptides folding in on themselves, side reactions to make the N-terminal amino acid unavailable for

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further coupling, steric hindrance, premature cleavage from the bead, and the like.

16. To fully understand the magnitude of the purification problems presented by the use of this type of synthetic chemistry, one needs to consider that all three of the foregoing problems, incomplete coupling, premature termination, and racemization, are occurring simultaneously. The result often is a wide variety of incorrect peptides, frequently including two or more of the aforementioned errors. The frequency of these errors, and therefore the degree of impurity, increases exponentially with the length of desired peptide. Proteins such as hPTH, which is 84 amino acids in length, are considered to be long and difficult to make synthetically, even by today's sophisticated standards. The technique employed by *Kumagaye et al*, cation exchange-HPLC, could be used for separating some of the resulting impurities. However, this technique would only work for that fraction of the total impurities having a charge differential when compared to native hPTH; a relatively minor percentage of the total impurities. In addition, depending upon the conditions used, not all of the differently charged species will be separated. Some of the resulting impurities may have a charge which is very similar to native hPTH, and may co-elute with hPTH. Any single impurity, if known, could theoretically be removed from the mixture by HPLC, RP-HPLC, and/or some other separation technique(s). Here, with the many possible impurities, it would be nearly impossible to effectively remove them all.

17. Persons familiar with cation exchange-HPLC would realize that the types of impurities to which I have referred result, almost inevitably, from the use of solid phase BOC

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chemistry and that many of the impurities co-elute with intact hPTH. Therefore, one would conclude, as I concluded, that the hPTH material resulting from the protocol described in *Kumagaye et al.* is pure only insofar as the one disclosed point mutant. No further conclusions about purity can be made. If anything, the impurities known to result from the solid phase synthesis described in the references would lead to the conclusion that the hPTH resulting from *Kumagaye et al.* would contain other impurities. *Kumagaye et al.* provide no explicit recitations of purity, provide no other form of characterization of the quality or quantity of the resulting hPTH material and provide nothing with regard to biological activity. Due to the cellular editing mechanisms found in, for example, yeast and *E. coli*, such impurities would not occur.

18. My opinion of all of the references, and, in particular *Kumagaye et al.*, is strengthened by the comparisons that I have reviewed between recombinant hPTH manufactured as described in the '664 application and commercially available, synthetically produced, hPTH produced by solid phase peptide synthesis.

19. As stated in paragraph 6, I have reviewed the declaration of Dr. Kaare M. Gautvik and, in particular, the photographs labeled Glossy 0 through Glossy III attached in Exhibits B-E, respectively, thereto. I understand from Dr. Gautvik's declaration that the materials analyzed and depicted in these photographs were made pursuant to the techniques described in the '664 application. Having reviewed that specification, I have no reason to question that assertion. The photographs are particularly informative because they provide a direct comparison between peptides produced by solid

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phase chemical synthesis and recombinant technology as described in the '664 application.

20. Glossy 0 illustrates an electrophoretic gel comparing recombinantly produced hPTH from Dr. Gautvik's laboratory with hPTH produced by solid phase synthesis sold by Sigma. Lane 2 (second from the left) contains the recombinant hPTH produced by Dr. Gautvik. The single broad band indicates homogeneity. In contrast, the Sigma material illustrated in Lane 3 shows a band migrating at roughly the same position as the hPTH produced recombinantly and two additional impurities of higher molecular weight. Based on the presence of these impurities in the Sigma material, the intensity and breadth of the bands and the relative intensities and sizes of the bands of hPTH, it is not hard to see that the recombinant material is orders of magnitude purer than the Sigma material.

21. Glossy III shows molecular weight standards in Lane S as well as recombinant hPTH produced from *E. coli* (Lane 4) and yeast (Lane 2) produced in accordance with the procedures outlined in the '664 application. Disposed between these materials, in Lane 3 is a synthetic material produced by solid phase synthesis available from a second chemical supplier, Bachem. The recombinant material is characterized by a single, sharp, dark, broad band corresponding to hPTH. In contrast, the Bachem lane illustrates the presence of lower molecular weight impurities in a smear. Moreover, the difference in the intensity of the staining indicates a significantly greater amount of hPTH in the recombinant preparations than in the chemically synthesized preparations, using an identical load (800 nanograms) of assayed material.

22. Glossy II contains, in addition to the information illustrated in Glossy III (lanes 27 through 29), identical preparations at a loading of 200 nanograms, (lanes 22 through 24). The difference in the intensities of the bands between the recombinantly produced material and the solid phase synthetic material available from Bachem illustrates the significantly greater amount of hPTH in the recombinant material, per unit weight. (Lanes 22 and 24 contain recombinant material and line 23 contains synthetic.) This information is totally consistent with the HPLC, N-terminal amino acid sequencing, mass spectrometry and two dimensional gel electrophoresis described in the '664 application. Based on this information, one of any level of skill in this art would conclude, as I have, that the recombinant material produced in accordance with the present invention is essentially pure.

23. My opinions are both verified and amplified by my review of Dr. Gautvik's declaration and, in particular, his publication in the peer-refereed journal *Peptides,* attached to his Declaration as Exhibit F. This article clearly demonstrates the biological properties of the recombinant material produced in accordance with the '664 application and verifies that which would be implicitly understood therefrom; namely that the hPTH material of the invention has biological activity substantially equivalent to naturally occurring human parathyroid hormone. Chemically synthesized material does not.

24. Dr. Gautvik's *Peptides* article is significant in that it illustrates both *in vivo* and *in vitro* biological activity. The results consistently reemphasize the superiority of the recombinant hPTH material in direct side-by-side comparisons to synthetic material. Fig. 1 of the *Peptides*

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paper illustrates the differences in binding affinity between recombinant hPTH produced in accordance with the '664 application from both *E. coli* and yeast and synthetic material from Bachem. The K_d of the recombinant material was 9.5nM while the K_d of the Bachem material was 18nM. This illustrates that the recombinant material contains approximately twice as much authentic hPTH when compared to the chemically synthesized material. The differences between these K_d values are very statistically significant as described in the paper (95% confidence intervals and redundant testing in triplicate). Because the K_d values indicate a greater amount of authentic hPTH per unit weight, the significant difference between the two K_d values indicate a dramatic difference in purity.

25. Fig. 2 of the *Peptides* paper illustrates the abilities of different preparations of hPTH to elicit a biological response in cell cultures. From Fig. 2 one can determine both an EC_{50} for cyclic AMP (cAMP) as well as a measure of efficacy or maximal response. EC_{50} is a measure of the potency of the materials in question. Here, the EC_{50} for recombinant hPTH is 1.5nM. The Ec_{50} for the Bachem material was 5.7nM. This is almost a four fold difference. As the figure and the accompanying text illustrate, this difference is highly statistically significant.

26. One of the more surprising findings outlined in the *Peptides* paper is the efficacy of the resulting materials. It appears that the Bachem material is only about 70% as efficacious as the recombinant hPTH. This means that no dose of synthetic material would be able to produce the maximal response of the tested cells, a problem not shared by the recombinant material. These two figures, acting in

combination, illustrate that the recombinant material is superior not only in purity, but also in binding and in eliciting a biological response. Moreover, the data just described are completely consistent with the data illustrated in Figs. 3-5 which show the *in vivo* activity of recombined hPTH in rats. In particular, Fig. 3 confirms the efficacy and potency data described in Fig. 2 by exhibiting an increased level of blood calcium over that achievable through the use of the synthetic material. Even 2.7 micrograms of Bachem PTH was unable to produce the same results as 2.0 micrograms of recombinant PTH.

27. The *Peptide* paper is a particularly good comparison of the recombinant material produced in accordance with the '664 application and synthetically produced material because of its careful characterizations and the variety of analytical techniques used. For example, peptide concentrations were determined by amino acid analysis. This is the premier method of determining peptide concentrations to date and is superior to other techniques such as optical density or dry weight. *In vivo* activity was measured not in one system, but rather by induction of hypercalcemia, urinary excretion of phosphate and by changes in urinary cAMP after administration of hPTH. Moreover, *in vitro* activity was assayed by receptor binding and cAMP responses of cells in culture. The variety of techniques used would appear to conclusively establish the superiority of recombinant material over synthetic material, both biologically and in terms of its purity. For these reasons, I believe that essentially pure recombinant material results from the practice of the invention described in the above-captioned application and that this hPTH

material is superior to anything in the prior art. I also believe that one of ordinary skill in the art would, upon reading the application, conclude as I have.

28. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: 6 MARCH 1996

JOHN E. MAGGIO, Ph.D.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of	:	
Gautvik et al..	:	
	:	Group Art Unit: 1812
Serial No. 08/340,664	:	
	:	Examiner: L. Spector
Filed: November 16, 1994	:	
	:	
For: PRODUCTION OF HUMAN	:	
PARATHYROID HORMONE FROM	:	
MICROORGANISMS	:	
	X	

Assistant Commissioner for Patents
Washington, D. C. 20231

DECLARATION OF JOHN E. MAGGIO, Ph.D. PURSUANT TO
37 C.F.R. § 1.132

Sir:

I, John E. Maggio, declare as follows:

1. I am a citizen of the United States of America residing at 480 Washington Street, Brookline, Mass. 02146.

2. I am an Associate Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School, Boston, Massachusetts. I have been a professor at Harvard Medical School since 1985.

3. My formal education includes Bachelors, Masters and Doctoral degrees, in the fields of chemistry and organic chemistry, all from Harvard University. I have also had extensive post-doctoral training at the University Chemical Laboratory and MRC Neurochemical Pharmacology Unit, Cambridge University, Cambridge, England, and at the Neuropsychopharmacology Research Unit at Yale University Medical School in New Haven. Both post-doctoral positions involved protein and peptide chemistry and purification thereof. My current curriculum vitae, including a list of my publications is attached as Exhibit 1.

4. My past and present work centers around synthesis, purification and characterization of biologically active peptides including tachykinins, magainins and amyloid peptides. As part of my work and since at least the late 1970s, I have used various forms of chromatography and electrophoresis for the purification of peptides and for their characterization. I am fully familiar with these techniques, and the state of their development throughout the 1980s and 1990s. I am also fully familiar with the past and present capabilities and limitations of such techniques. Representative of my work with peptide separation and characterization is an article attached as Exhibit 2 entitled "Mapping Peptide-binding Domains of the Substance P(NK-1) Receptor from P388D$_1$ Cells with Photolabile Agonists", *J. Biological Chemistry.* 270, (1995), 1213-1220.

5. My knowledge of chromatography, electrophoresis, and other techniques used commonly in protein chemistry stems from my repeated use of those techniques throughout my career. I have supervised students and other scientists using these techniques and have taught the techniques, both in the classroom and in the laboratory. Therefore, I am comfortable judging the ordinary level of skill that a person in this art would possess in terms of the theoretical and the bench aspects of these techniques.

6. In preparing this declaration I reviewed, among other things, the following materials: a copy of the specification of U.S. Serial No. 08/340,664, filed November 16, 1994, (the "'664 application"); a copy of the Official Action dated September 8, 1995 issued by the patent examiner, Dr. Spector; copies of each of the references identified in the

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Official Action; and a copy of the Declaration of Kaare M. Gautvik, M.D. Pursuant to 37 C.F.R. § 1.132 as well as the documents and photographs attached thereto.

7. I understand from my review of the Official Action, the United States Patent and Trademark Office has refused to grant the '664 application in view of the disclosures of four references: *Brewer et al., Fairwell et al., Kimura et al.* and/or *Kumagaye et al.*

8. I have reviewed the four references cited by the Patent Office and I do not agree with the Patent Office's conclusions regarding their teachings or disclosures. In my opinion, none of the references describe or suggest a method of obtaining a substantially pure, intact, hPTH peptide. None of the references provides a basis for concluding that a substantially pure hPTH product was actually produced. Further, nothing in the references describes an hPTH peptide having biological activity substantially equivalent to naturally occurring hPTH. I believe that a biochemist, organic chemist or analytical chemist having an ordinary level of skill in this technology would not be unable to draw any conclusion with regard to the purity of hPTH produced in accordance with the cited references. If anything, given the errors appearing in those references and the known shortcomings of the techniques described in the references, *e.g.* solid phase chemical synthesis, those of ordinary skill in the art would probably assume that the resulting hPTH material was impure.

9. *Brewer et al.* relate to an isolation from tissue, not a recombinant material. *Brewer et al.* contain three errors at positions 22, 28 and 30 of the synthesized peptide compared to the wild-type peptide. This is illustrated

in Fig. 1 of *Brewer et al.* Accordingly, *Brewer et al.* do not teach the production of an *intact* hPTH peptide. Moreover, two later publications cited by Dr. Spector in the Official Action, namely *Kimura et al.* and *Kumagaye et al.*, show that the purification protocols discussed in *Brewer et al.* result in impure materials. For example, Fig. 2, on page 496 of *Kimura et al.* is an HPLC profile of crude product obtained after use of a separation protocol analogous to that disclosed in *Brewer et al.*; namely, the use of a combination of gel filtration and ion exchange chromatography. Impurities are plainly evident. Therefore, a conclusion of homogeneity based on *Brewer et al.* is unjustified. Further, *Kimura et al.* describe a purification sequence of CM-cellulose column chromatography followed by gel filtration on Sephadex G-50, followed in turn by the use of reverse phase-high pressure liquid chromatography ("RP-HPLC"). *Kimura et al.* added the RP-HPLC step in recognition of the need to obtain better purity than *Brewer et al.* obtained. This fact alone, in my opinion, eliminates any plausible basis for concluding that the protein resulting from the methods described in *Brewer et al.* was essentially pure.

10. Many of the criticisms of *Brewer et al.* apply to *Fairwell et al.* For example, *Fairwell et al.* produced a peptide having an Asp at position 76. Native hPTH has an Asn in that position. *Fairwell et al.* also used a separation protocol combining the use of gel filtration and ion exchange chromatography. As previously mentioned, that protocol was re-run by *Kimura et al.* and the results, as illustrated in the chromatogram in Fig. 2 thereof, show significant impurities. Finally, as *Kimura et al.* used RP-HPLC rather than relying

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merely on the separation protocol described in *Fairwell et al.*, it is clear that subsequent investigators believed that the separation protocols of *Fairwell et al.* were inadequate.

11. *Kimura et al.* did not produce an essentially pure hPTH. As *Kumagaye et al.* clearly explain, "[t]oday, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products." (Emphasis added) *Kumagaye et al.* at page 330. This is especially significant because *Kumagaye et al.* is the same group of researchers as *Kimura et al.*

12. *Kumagaye et al.* disclose a method of separating two different forms of hPTH from a mixture thereof by using cation exchange-HPLC. This is not a particularly surprising result as the two forms of hPTH disclosed have a full charge difference between them, a situation ideal for the use of cation exchange-HPLC. Nonetheless, one of ordinary skill in the art would not conclude that the resulting hPTH in accordance with *Kumagaye et al.* was essentially pure. One could conclude that the resulting material was pure of the one specific impurity, i.e. the specific point mutated form disclosed. However, there is no basis for concluding further. If anything, as explained herein, there is every reason to believe that impurities are present.

13. *Kumagaye et al.* describe a solid phase peptide synthesis protocol which was common at the time. That synthesis, as explained in the *Kimura et al.* article, involved the use of BOC protected amino acids and traditional BOC chemistry. Using BOC chemistry, each successive amino acid is

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added to the N-terminus of a growing chain by first removing the blocking group in acid, then neutralizing prior to coupling, followed by coupling the next BOC amino acid in sequence using, for example, dicyclohexylcarbodiimide ("DCC").

14. This technology suffers from a number of well known shortcomings and, in fact, has largely been replaced. One of the better known and most common problems with solid phase BOC chemistry, particularly for longer peptides such as hPTH, is racemization. As explained in *Bodanszky, "Peptide Chemistry; A Practical Textbook"* at page 120, the problem of racemization using DCC coupling and BOC chemistry is well documented. *See* Exhibit 3. This chapter, as well as the others attached as Exhibit 3, demonstrate the prevalence of racemization and concerns over this phenomenon during solid phase protein synthesis.

15. Some of the other well known impurities generated by solid phase synthesis are described in the *Fairwell et al.* article cited by Dr. Spector at page 2691. These impurities include, among other things, deletion peptides, omission peptides and prematurely terminated peptides. For example, during solid phase synthesis it is possible for coupling to be either duplicative or incomplete, thereby providing a peptide having an additional amino acid or an omission from the normal sequence. There may be one or more additions and/or deletions in any given peptide. These additions and/or deletions can occur almost anywhere along the chain. Premature termination of the chain length is also common. This may occur for a host of reasons such as, peptides folding in on themselves, side reactions to make the N-terminal amino acid unavailable for

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further coupling, steric hindrance, premature cleavage from the bead, and the like.

16. To fully understand the magnitude of the purification problems presented by the use of this type of synthetic chemistry, one needs to consider that all three of the foregoing problems, incomplete coupling, premature termination, and racemization, are occurring simultaneously. The result often is a wide variety of incorrect peptides, frequently including two or more of the aforementioned errors. The frequency of these errors, and therefore the degree of impurity, increases exponentially with the length of desired peptide. Proteins such as hPTH, which is 84 amino acids in length, are considered to be long and difficult to make synthetically, even by today's sophisticated standards. The technique employed by *Kumagaye et al*, cation exchange-HPLC, could be used for separating some of the resulting impurities. However, this technique would only work for that fraction of the total impurities having a charge differential when compared to native hPTH; a relatively minor percentage of the total impurities. In addition, depending upon the conditions used, not all of the differently charged species will be separated. Some of the resulting impurities may have a charge which is very similar to native hPTH, and may co-elute with hPTH. Any single impurity, if known, could theoretically be removed from the mixture by HPLC, RP-HPLC, and/or some other separation technique(s). Here, with the many possible impurities, it would be nearly impossible to effectively remove them all.

17. Persons familiar with cation exchange-HPLC would realize that the types of impurities to which I have referred result, almost inevitably, from the use of solid phase BOC

chemistry and that many of the impurities co-elute with intact hPTH. Therefore, one would conclude, as I concluded, that the hPTH material resulting from the protocol described in *Kumagaye et al.* is pure only insofar as the one disclosed point mutant. No further conclusions about purity can be made. If anything, the impurities known to result from the solid phase synthesis described in the references would lead to the conclusion that the hPTH resulting from *Kumagaye et al.* would contain other impurities. *Kumagaye et al.* provide no explicit recitations of purity, provide no other form of characterization of the quality or quantity of the resulting hPTH material and provide nothing with regard to biological activity. Due to the cellular editing mechanisms found in, for example, yeast and *E. coli*, such impurities would not occur.

18. My opinion of all of the references, and, in particular *Kumagaye et al.*, is strengthened by the comparisons that I have reviewed between recombinant hPTH manufactured as described in the '664 application and commercially available, synthetically produced, hPTH produced by solid phase peptide synthesis.

19. As stated in paragraph 6, I have reviewed the declaration of Dr. Kaare M. Gautvik and, in particular, the photographs labeled Glossy 0 through Glossy III attached in Exhibits B-E, respectively, thereto. I understand from Dr. Gautvik's declaration that the materials analyzed and depicted in these photographs were made pursuant to the techniques described in the '664 application. Having reviewed that specification, I have no reason to question that assertion. The photographs are particularly informative because they provide a direct comparison between peptides produced by solid

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phase chemical synthesis and recombinant technology as described in the '664 application.

20. Glossy 0 illustrates an electrophoretic gel comparing recombinantly produced hPTH from Dr. Gautvik's laboratory with hPTH produced by solid phase synthesis sold by Sigma. Lane 2 (second from the left) contains the recombinant hPTH produced by Dr. Gautvik. The single broad band indicates homogeneity. In contrast, the Sigma material illustrated in Lane 3 shows a band migrating at roughly the same position as the hPTH produced recombinantly and two additional impurities of higher molecular weight. Based on the presence of these impurities in the Sigma material, the intensity and breadth of the bands and the relative intensities and sizes of the bands of hPTH, it is not hard to see that the recombinant material is orders of magnitude purer than the Sigma material.

21. Glossy III shows molecular weight standards in Lane S as well as recombinant hPTH produced from *E. coli* (Lane 4) and yeast (Lane 2) produced in accordance with the procedures outlined in the '664 application. Disposed between these materials, in Lane 3 is a synthetic material produced by solid phase synthesis available from a second chemical supplier, Bachem. The recombinant material is characterized by a single, sharp, dark, broad band corresponding to hPTH. In contrast, the Bachem lane illustrates the presence of lower molecular weight impurities in a smear. Moreover, the difference in the intensity of the staining indicates a significantly greater amount of hPTH in the recombinant preparations than in the chemically synthesized preparations, using an identical load (800 nanograms) of assayed material.

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22. Glossy II contains, in addition to the information illustrated in Glossy III (lanes 27 through 29), identical preparations at a loading of 200 nanograms, (lanes 22 through 24). The difference in the intensities of the bands between the recombinantly produced material and the solid phase synthetic material available from Bachem illustrates the significantly greater amount of hPTH in the recombinant material, per unit weight. (Lanes 22 and 24 contain recombinant material and line 23 contains synthetic.) This information is totally consistent with the HPLC, N-terminal amino acid sequencing, mass spectrometry and two dimensional gel electrophoresis described in the '664 application. Based on this information, one of any level of skill in this art would conclude, as I have, that the recombinant material produced in accordance with the present invention is essentially pure.

23. My opinions are both verified and amplified by my review of Dr. Gautvik's declaration and, in particular, his publication in the peer-refereed journal *Peptides,* attached to his Declaration as Exhibit F. This article clearly demonstrates the biological properties of the recombinant material produced in accordance with the '664 application and verifies that which would be implicitly understood therefrom; namely that the hPTH material of the invention has biological activity substantially equivalent to naturally occurring human parathyroid hormone. Chemically synthesized material does not.

24. Dr. Gautvik's *Peptides* article is significant in that it illustrates both *in vivo* and *in vitro* biological activity. The results consistently reemphasize the superiority of the recombinant hPTH material in direct side-by-side comparisons to synthetic material. Fig. 1 of the *Peptides*

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paper illustrates the differences in binding affinity between recombinant hPTH produced in accordance with the '664 application from both *E. coli* and yeast and synthetic material from Bachem. The K_d of the recombinant material was 9.5nM while the K_d of the Bachem material was 18nM. This illustrates that the recombinant material contains approximately twice as much authentic hPTH when compared to the chemically synthesized material. The differences between these K_d values are very statistically significant as described in the paper (95% confidence intervals and redundant testing in triplicate). Because the K_d values indicate a greater amount of authentic hPTH per unit weight, the significant difference between the two K_d values indicate a dramatic difference in purity.

25. Fig. 2 of the *Peptides* paper illustrates the abilities of different preparations of hPTH to elicit a biological response in cell cultures. From Fig. 2 one can determine both an EC_{50} for cyclic AMP (cAMP) as well as a measure of efficacy or maximal response. EC_{50} is a measure of the potency of the materials in question. Here, the EC_{50} for recombinant hPTH is 1.5nM. The EC_{50} for the Bachem material was 5.7nM. This is almost a four fold difference. As the figure and the accompanying text illustrate, this difference is highly statistically significant.

26. One of the more surprising findings outlined in the *Peptides* paper is the efficacy of the resulting materials. It appears that the Bachem material is only about 70% as efficacious as the recombinant hPTH. This means that no dose of synthetic material would be able to produce the maximal response of the tested cells, a problem not shared by the recombinant material. These two figures, acting in

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combination, illustrate that the recombinant material is superior not only in purity, but also in binding and in eliciting a biological response. Moreover, the data just described are completely consistent with the data illustrated in Figs. 3-5 which show the *in vivo* activity of recombined hPTH in rats. In particular, Fig. 3 confirms the efficacy and potency data described in Fig. 2 by exhibiting an increased level of blood calcium over that achievable through the use of the synthetic material. Even 2.7 micrograms of Bachem PTH was unable to produce the same results as 2.0 micrograms of recombinant PTH.

27. The *Peptide* paper is a particularly good comparison of the recombinant material produced in accordance with the '664 application and synthetically produced material because of its careful characterizations and the variety of analytical techniques used. For example, peptide concentrations were determined by amino acid analysis. This is the premier method of determining peptide concentrations to date and is superior to other techniques such as optical density or dry weight. *In vivo* activity was measured not in one system, but rather by induction of hypercalcemia, urinary excretion of phosphate and by changes in urinary cAMP after administration of hPTH. Moreover, *in vitro* activity was assayed by receptor binding and cAMP responses of cells in culture. The variety of techniques used would appear to conclusively establish the superiority of recombinant material over synthetic material, both biologically and in terms of its purity. For these reasons, I believe that essentially pure recombinant material results from the practice of the invention described in the above-captioned application and that this hPTH

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material is superior to anything in the prior art. I also believe that one of ordinary skill in the art would, upon reading the application, conclude as I have.

28. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: 6 March 1996 _____

_____
JOHN E. MAGGIO, Ph.D.

For: PRODUCTION OF HUMAN
    PARATHYROID HORMONE FROM   :
    MICROORGANISMS   :
                                  :
_____X

Assistant Commissioner for Patents
Washington, D.C. 20231

<u>DECLARATION OF KAARE M. GAUTVIK, M.D.</u>
<u>PURSUANT TO 37 C.F.R. § 1.132</u>

Sir:

        I, KAARE M. GAUTVIK, declare as follows:

        1.   I am a coinventor of the above-captioned
application.

        2.   I am a citizen of Norway residing at Bregnevn 3,
0875 Oslo, Norway. I am fluent in English. My curriculum vitae
is attached hereto as exhibit A.

        3.   Throughout the 1980's, and continuing today, I
have had a keen interest in a number of medical conditions
including osteoporosis. In the early 1980's not much was known
regarding this condition. As best exemplified by the *Brewer et
al.* patent cited by the Examiner in the Official Action dated
September 8, 1995, most of the emphasis at the time was on the
N-terminal region, that could bind to certain receptor cells in
bones. But the hPTH peptide was not well characterized and
certain phenomena could not be explained by this binding. I and
others sought to explain these hitherto unexplained phenomena.
To test various theories, a good, inexpensive supply of very
pure hPTH was needed. At the time, the only way to obtain hPTH
was by extraction and isolation, followed by complex
purification from human tissue. This was more than just a
laborious process. Due to the difficulty in obtaining human
tissue where hPTH had not deteriorated, relatively little
material could be extracted and isolated at any one time.

this was accomplished, purification of the expressed protein could be carried out using the technologies that were prevalent at the time. I therefore sought the skills of my coinventors, and together we developed a source of raw material. The manner in which that was accomplished, and the resulting highly pure peptide, is described my above-captioned patent application.

5. By the use of recombinant technology as described in the patent application, we have been able to obtain hPTH which is not only of significantly higher purity than anything otherwise available, but also hPTH which was qualitatively superior. The data presented herein describing the attributes of the essentially pure, recombinant hPTH we developed are based on hPTH hormone produced by me or under my direct supervision in the mid to late 1980s. The resultant peptide was purified as described in the application. No other purification steps were employed.

6. Attached as Exhibits B-E are a number of glossy photographs labeled Glossy 0 through Glossy III. These glossies contain, among other things, photographs of electrophoresis gels run by me or under my direct supervision. Glossy 0 (Exhibit B) corresponds to an electrophoretic gel comparing synthetic hPTH obtained from the chemical supply company, Sigma, to recombinant hPTH obtained from yeast as disclosed in the above-captioned application. This gel was prepared before the filing of my patent application which issued as U.S. Patent No. 5,420,242. Lanes 1 and 4 contain chemical markers. Lane 2 (second from the left) contains recombinant hPTH prepared in accordance with the present invention. The Sigma material was loaded in Lane 3 (third from the left). The symmetrical blurring on either side of the actual hPTH band is the result of overloading the gel. It is significant to note that a single band of material is present in Lane 2 while three distinct bands are found in

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material were run (Lot Nos. 2E567 and 7345). Gels were loaded with either 200 nanograms or 800 nanograms of material as indicated, according to the manufacturers instructions. These materials were run against three lanes with molecular markers (Lanes 2, 11 and 20) as well as our recombinant hPTH produced from *E. coli* (Lanes 8 and 17), yeast (Lanes 9 and 18) and QPTH (Lanes 10 and 19). The materials obtained from Peptide and from Peninsula ran as a higher molecular species of much less quantity than indicated by the manufacturer and no correctly sized hPTH could be seen in the Peptide lanes, even when applied at 800 nanograms. The Peninsula material in Lane 4 shows a small indication of correct hPTH, but most of the material exists as a high molecular weight form. The Sigma preparation ran at a correct location but contained much less material than the manufacturer indicated. No material was evident at a loading of 200 nanograms. The two different batches from Bachem show a peptide of correct molecular size, but one of the preparations shows a heavy, and the other a lesser, trailing smear indicating lower molecular weight impurities. Again, the amounts of hTPH contained in the Bachem bands appear to be less than the amounts contained in the bands corresponding to the same loaded amount of recombinant hPTH from yeast and *E. coli*. Each of the three recombinant hPTHs appear as very sharp, fat bands, of equal intensity and much stronger intensity than any of the chemically synthesized preparations. When applied as 200 nanograms, only the recombinant hPTH lanes can be clearly seen. Everyone familiar with gel electrophoresis of protein knows that as little as 100 nanograms is usually sufficient to provide detectable staining. Thus, the absence of staining of 200 nanograms and diffuse bands at 800 nanograms are indicative of a relatively impure peptide.

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versus 23 and 27 versus 28, respectively). The Bachem preparation, shown in Lanes 23 and 28 shows considerable trailing toward degradation products. The amount of the correct material of the Bachem preparation may best be judged by the electrophoresis of the 200 nanogram sample. At 200 nanograms of material loaded, very little Bachem material was observed and lower molecular weight species are seen as a trailing area.

9. Glossy III (Exhibit E) is a blow-up of Glossy II indicating the size of the molecular markers in the first lane on the left. We have since carried out more recent electrophoresis and the bands appear exactly as they did in 1988 and 1989, indicating that there was no degradation of our preparation since its production in the late 1980's. The absence of degradation also indicates the substantial purity of the resulting material. The foregoing clearly indicates the superior purity of the material resulting in accordance with the present invention. However, it is also my opinion that because the hPTH produced in accordance with the present invention is recombinant material purified as explained in the application, not only is the peptide of better purity, but it is also of a significantly better quality. The differences are aptly illustrated in some of my prior published works.

10. For example, attached hereto as Exhibit F is a copy of my paper, "Differences in Binding Affinities of Human PTH(1-84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms," published in the refereed journal, *Peptides*, (1994), 15, 1261-65. The data reported in this paper involved the analysis of hPTH material produced in accordance with the patent application in the mid to late 1980's. In fact, no other purification steps were taken, other than those disclosed in the patent application prior to the analyses described in this paper.

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at least two independent experiments each performed in triplicate. As will be self evident from the figure, chemically synthesized hPTH had a calculated binding affinity, (K_d) of 18nM (95% confidence interval: 16.1-20.0nM) while recombinant hPTH (1-84) from both yeast and *E. coli* had a significantly lower apparent K_d of 9.5nM (95% confidence interval: 8.7-10.4nM).

12. Fig. 2 illustrates the stimulation of cAMP by different types of hPTH. The recombinant hormones in accordance with the present invention have the ability to stimulate intercellular cAMP accumulation with an EC_{50} of about 1.5nM, (95% confidence interval 1.0-2.2nM). In contrast, the solid phase synthesized hPTH showed a significantly reduced potency in stimulating cAMP production with an EC_{50} value of 5.7nM (95% confidence interval: 3.4-9.6nM). Fig. 2 also illustrates that the synthetically produced hPTH exhibited a reduced maximal response. Therefore, no matter how much synthetic hPTH is administered, it is not possible to obtain the same efficacy as that obtained by the administration of recombinant peptides in accordance with the present invention. These same results were mirrored in the *in vivo* testing undertaken and illustrated in Figs. 3 through 5.

13. A common way to measure hPTH bioactivity is to determine its ability to activate cell membrane-bound adenylate cyclase in target cells, e.g., bone derived cells. When hPTH binds to its receptor, adenylate cyclase is activated. This generates cAMP from ATP Mg. The activity of adenylate cyclase can be directly measured in membrane fractions of broken target cells when radioactive ATP Mg is added and the radioactively generated cAMP is isolated and quantitated by scintilation counting. The formation of cAMP

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amount of cAMP generated inside intact target cells have been treated with hPTH in the presence of an inhibitor of cAMP degradation. After a certain period of treatment, the reaction is stopped (the cells are killed) and cAMP is measured after extraction by radioimmunoassay. This is the test described in my paper in the journal *Peptides* (Fig. 2). Thus, the adenylate cyclase and the cAMP assays, both individually and collectively, establish the full biologicol activity of the hPTH I and my coinventors made.

14. I have been warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon. I declare that all statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true.

Dated: _02,29,96_   _Kaare M. Gautvik_
                           KAARE M. GAUTVIK, M.D.

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☐Citation 5

Unique Identifier
    81124291
Authors
    Gordon DF. Kemper B .
Title
    Synthesis, restriction analysis, and molecular **cloning** of near full length DNA **complementary** to **bovine parathyroid** hormone mRNA.
Source
    Nucleic Acids Research. 8(23):5669-83, 1980 Dec 11.
Abstract

    DNA **complementary** (cDNA) to a partially purified preparation of **bovine parathyroid** hormone mRNA was synthesized using avian myeloblastosis viral reverse transcriptase. The PTH cDNA contained about 750 bases and was greater than 95% sensitive to digestion by S1 nuclease. Analysis of the mRNA preparation by excess RNA hybridization to the PTH cDNA revealed one rapidly hybridizing component consisting of 50% of the PTH cDNA. Sequential incubation of the PTH mRNA with reverse transcriptase and E. coli DNA polymerase I produced near full length double-stranded PTH cDNA. Of the 22 restriction endonucleases tested, double-stranded PTH cDNA could be cleaved with Alu I, Mbo II, Sau 3A, Sst I, and Taq I. The restriction fragments corresponding to the 5' terminus of the sense strand were identified for the last three enzymes by comparing the size of fragments obtained from PTH cDNA before and after cleavage of the hairpin loop connecting the two strands by S1 nuclease. The restriction map of the cDNA was used to detect clones of bacteria containing recombinant plasmids with near full length PTH cDNA inserts.

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GLOSSY 3
EXHIBIT E

GLOSSY 2
EXHIBIT D

GLOSSY 1
EXHIBIT C

GLOSSY 0
EXHIBIT B

Human Parathyroid Hormone Gene (*PTH*) Is on Short Arm of Chromosome 11

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Abstract—*The human gene for parathyroid hormone (PTH) was chromosomally mapped using human-rodent hybrids and Southern filter hybridization of cell hybrid DNA. A recombinant DNA probe containing human PTH cDNA insert (pPTHm122) hybridized to a 3.7-kb fragment in human DNA cleaved with the restriction enzyme EcoRI. By correlating the presence of this fragment in somatic cell hybrid DNA with the human chromosomal content of the hybrid cells, the PTH gene was mapped to the short arm of the chromosome 11.*

INTRODUCTION

Parathyroid hormone (PTH) is a polypeptide hormone which is a major regulator of calcium in the blood (1). Bovine and human PTH are produced as precursor molecules (preproPTH), and cDNA clones derived from mRNA have been isolated and sequenced for both species (2, 3). To determine the location of the *PTH* gene in the human genome, we chromosomally mapped the gene by Southern filter hybridization of human–rodent somatic cell hybrid DNA. Since these human–rodent hybrids segregate human chromosomes, human genes are mapped by correlating a specific human chromosome with the presence of the human gene (4).

MATERIALS AND METHODS

Hybrid Cell Lines. Human–mouse somatic cell hybrids were made by fusion of parental cells with polyethylene glycol (5) and were isolated on HAT

selection medium (6). Each of the hybrid series used has been described in previous publications: NSL (GM2836 × LMTK⁻) (7), ATR (AlTr × RAG) (8), JSR (JoSt × RAG) (9), XER (GM2859 × RAG) (10), EXR (GM 3322 × RAG) (10), WIL (WI-38 × LTP) (11), REW (WI-38 × RAG) (11), and ICL (GM1006 × LMTK⁻) (12). Some of the hybrid lines segregate human translocation chromosomes characteristic of the human parental line. These translocations are indicated in the table legends.

Human Chromosome Composition of Cell Hybrids. Hybrid cells were karyotyped (13) and/or analyzed for genetic markers previously assigned to each human autosome and the X chromosome to determine their human chromosome content. The markers used in this study are: chromosome 1, peptidaseC (PEPC) and adenylate kinase-2 (AK2); 2, malate dehydrogenase (soluble) (MDH1) and isocitrate dehydrogenase (soluble) (IDH1); 3, amino-acylase-1 (ACY1); 4, peptidase-S (PEPS); 5, β-hexosaminidase-A (HEXA); 6, malic enzyme (soluble) (ME1); 7, β-glucuronidase (GUSB); 8, glutathione reductase (GSR); 9, adenylate kinase-1 (AK1), aconitase (soluble) (ACO1); 10, glutamate oxaloacetate transaminase (soluble) (GOT1); 11, lactate dehydrogenase-A (LDHA) and esterase-A₄ (ESA4); 12, lactate dehydrogen-ase-B (LDHB) and peptidase-B (PEPB); 13, esterase-D (ESD); 14, nucleo-side phosphorylase (NP); 15, mannose phosphate isomerase (MPI) and pyruvate kinase (muscle form) (PKM2); 16, adenine phosphoribosyltransfer-ase (APRT); 17, galactokinase (GALK); 18, peptidase-A (PEPA); 19, glucose phosphate isomerase (GPI); 20, adenosine deaminase (ADA); 21, superoxide dismutase (soluble) (SOD1); 22, aconitase (mitochondrial) (ACO2); and X, glucose-6-phosphate dehydrogenase (G6PD) and phospho-glycerate kinase (PGK).

pPTHm122 probe. Human cDNA of preproparathyroid hormone was cloned in the PstI site of pBR322 (3). The 600-bp (base pair) insert contained all of the coding sequences for preproPTH as well as 3' and 5' flanking sequences.

Southern Filter Analysis of Cell Hybrid DNA. DNA was isolated from human, mouse, and hybrid cells as described (14). Ten micrograms of each DNA sample was cleaved with EcoRI (4 units/μg DNA) for 3 h at 37°C using the buffer recommended by the manufacturer. DNA fragments were sepa-rated by electrophoresis in 0.8% agarose gels and transferred onto nitrocellu-lose by the method of Southern (15). The pPTHm122 probe (3) was labeled with ³²P by nick translation using [³²P]dCTP and [³²P]dTTP (16) to a specific activity of 2 × 10⁸ cpm/μg. The probe was hybridized to filters by the method of Wahl (17) for 2 days at 42°C. The blots were rinsed briefly in 2X SSC, 0.1% SDS at room temperature, and then washed at least twice for 45 min in 0.1X SSC, 0.1% SDS at 50°C. The dried blots were exposed to Kodak XAR X-ray film at −70°C for 1–7 days using Dupont Cronex Lightning Plus intensifying screens.

PTH on Human Chromosome

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RESULTS

Detection of Human PTH Sequences. The plasmid pPTHm122 was used to detect human *PTH* DNA sequences in somatic cell hybrids. pPTHm122, a cDNA clone isolated from a human cDNA library of parathyroid tissue (3), contains a 600-bp insert which includes the entire preproPTH sequence (3). Human genomic DNA digested with the restriction endonuclease EcoRI and separated by agarose electrophoresis, yields a single 3.7-kb band upon Southern filter hybridization (15) with labeled pPTHm122 probe (Fig. 1). Under the conditions used for hybridization and washing, mouse PTH sequences are not detected. Southern filter hybridization of hybrid cell DNAs results in either the single human band or none, depending on the human chromosome composition of the hybrid cells.

Mapping Human PTH. The human chromosome content of the hybrid cells was determined by marker enzyme analysis in all cases and by direct karyotyping of several hybrid cells. Marker enzymes assigned to both the human autosomes and the X chromosome (4) give a reliable indication of the human chromosomes present in a hybrid cell. By correlating the presence of these markers with the detection of human PTH sequences in 20 hybrids, the gene for PTH was found to be present or absent together with the human isozymes of lactate dehydrogenase-A and esterase-A$_4$ (Table 1). Both of these genetic markers have been assigned to chromosome 11 (18–21). Southern

Fig. 1. Hybridization of the pPTHm122 probe to EcoRI-digested DNA from somatic cell hybrids. pPTHm122 hybridizes to a 3.7-kb EcoRI fragment of human DNA (H) but not to mouse (M) under these conditions. Cell hybrid DNAs digested with EcoRI either contain the human 3.7-kb fragment (+) or do not (−), depending on the human chromosome composition of the cells.

Table 1. Segregation of PTH with Human Enzyme Markers in Somatic Cell Hybrids[a]

Chromosome	Enzyme markers	PTH/enzyme marker				Discordancy (%)
		+/+	+/−	−/+	−/−	
1	PEPC, AK2	1	5	2	9	41
2	MDH1, IDH1	4	3	1	11	21
3	ACY1	4	4	2	10	30
4	PEPS	3	5	5	7	50
5	HEXA	3	5	6	6	55
6	ME1, SOD2	2	5	1	11	32
7	GUSB	3	5	3	9	40
8	GSR	3	3	4	8	39
9	AK1, ACO1	1	7	0	7	47
10	GOT1	7	1	5	7	30
11	LDHA, ESA4	8	0	0	12	0
12	LDHB, PEPB	6	2	6	6	40
13	ESD	4	4	2	10	30
14	NP	6	2	3	9	25
15	MPI, PKM2	1	7	6	5	68
16	APRT	3	5	4	8	45
17	GALK	7	1	11	1	60
18	PEPA	7	1	5	7	30
19	GPI	5	3	3	9	30
20	ADA	8	0	6	6	30
21	SOD1	7	1	5	7	32
22	ACO2	1	7	2	10	45
X	G6PD, PGK	5	3	2	10	25

[a]Twenty hybrids characterized for human chromosomes by marker enzymes were tested for human PTH sequences. Concordant hybrids (+/+, −/−) either had or lacked PTH and the specific marker enzymes, whereas +/− and −/+ indicate discordant hybrids. The results are summarized in the right-hand column as a percent discordancy. Enzyme scores were not included for those markers from chromosomes which were translocated in the human parental line as it was not always possible to discern whether the normal or translocated chromosome(s) was present.

filter hybridization was also done on DNA from nine additional hybrid lines which were karyotyped as well as tested for marker enzymes. The 3.7-kb band was detected only in those hybrid cells containing human chromosome 11 (Table 2) with no exceptions.

Regional Localization of PTH *Gene.* Subchromosomal localization of the *PTH* gene was accomplished by analyzing hybrids made from human cells containing translocations of human chromosome 11. Two sets of hybrids were constructed from the fusion of mouse RAG cells with human fibroblasts possessing two different translocations: GM2859 [46,Xdel(11)(p11p13)-t(X;11) (Xpter→Xq11::11p13→11pter; 11qter→11p11::Xq11→Xqter)] used in the XER hybrid series and GM3322 [46,X,t(X;11)(q22;q13)] in the EXR hybrids (10). XER-7 and XER-9 both contain the centromere and long arm of chromosome 11 (11p11→11qter) but do not contain PTH sequences

Table 2. Segregation of *PTH* with Human Chromosomes in Human–Mouse Hybrids[a]

Chromosomes	NSL-7	NSL-9	NSL-15	ATR-15	JSR-17S	XER-7	XER-9	EXR-5CSAz	EXR-3
1	−	−	−	+	+	+	−	+	+
2	−	−	+	+	+	+	+	−	+
3	−	−	−	+	−	+	−	+	+
4	−	−	+	+	+	+	+	+	+
PTH	−	−	−	−	+	−	−	−	+

omatic Cell Hybrids^a

−/−	Discordancy (%)
9	41
11	21
10	30
7	50
6	55
11	32
9	40
8	39
7	47
7	30
12	0
6	40
10	30
9	25
5	68
8	45
1	60
7	30
9	30
6	30
7	32
10	45
10	25

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Table 2. Segregation of *PTH* with Human Chromosomes in Human–Mouse Hybrids^a

Chromosomes	NSL-7	NSL-9	NSL-15	ATR-15	JSR-17S	XER-7	XER-9	EXR-5CSAz	EXR-3
1	−	−	−	−	−	−	−	−	+
2	−	−	+	+	+	+	+	+	+
3	−	−	−	+	−	+	−	−	+
4	−	−	+	+	−	+	+	+	+
5	−	+	+	−	+	+	−	+	+
6	−	−	+	+	−	+	−	+	+
7	+	−	+	+	−	+	−	+	+
8	−	+	−	+	−	−	+	+	−
9	−	−	+	+	+	−	−	+	+
10	−	+	−	−	+	+	+	+	+
11	+	−	+	+	+	+	−	+	+
12	+	+	+	+	+	+	+	+	+
13	−	+	+	−	+	+	+	−	+
14	−	+	+	+	+	+	−	+	+
15	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	−
17	−	−	+	−	+	−	+	+	+
18	−	+	+	+	−	−	+	+	+
19	−	+	+	−	+	+	+	+	+
20	+	−	+	+	+	−	−	+	−
21	+	+	+	−	+	−	+	+	+
22	−	+	+	−	+	+	−	+	+
X	−	−	+	−	−	+	−	+	−
Translocation chromosomes^b		17/9		5/X		11/X	11/X	X/11	X/11, 11/X
PTH	−	−	−	−	+	−	−	−	+

^a Hybrid cells were karyotyped to determine their human chromosome content as previously described (4). "+" indicates a presence of a given human chromosome in ≥10% of the metaphases scored. Marker enzymes were also tested for each hybrid cell.
^b Translocation chromosomes are from spontaneous translocations found in the individuals whose fibroblasts were used to form hybrid clones. NSL hybrids contain a 17/9 chromosome (17qter → 17p11::9p11 → 9pter); ATR hybrids a 5/X (5pter → 5q35::Xq22 → Xqter); XER hybrids: XER-9 hybrid 11/X (11qter → 11p11::Xqter); and EXR hybrids X/11 (Xpter → Xq22::11q13 → 11qter) and 11/X (11pter → 11q13::Xq22 → Xqter).

(Table 2). Likewise, EXR-5CSAz contains most of the long arm of chromo-
some 11 and also does not have the human *PTH* gene. These data suggest that
PTH is located on the short arm of chromosome 11. However, we examined
DNA from a hybrid derived from GM2859 that contains both translocation
chromosomes (X/11 and 11/X) and did not find hybridization to the human
PTH probe. The X/11 chromosome has been reported by others to have
deletion in the 11p11→11p13 region (22, and B. Zabel, personal communica-
tion). It is interesting to note that this cell hybrid does not have human insulin
sequences and also does not express human lactate dehydrogenase-A (our
unpublished observations). We would therefore assign the gene for PTH to the
short arm of chromosome 11 and suggest that it may be located in the
p11→p13 region.

Screening for DNA Polymorphisms with PTH Probe. PTH lies within
the subchromosomal segment of chromosome 11 which contains the β-globin
genes (23–26) and the human insulin gene (27–30). It would be of interest,
then, to do linkage studies with these genes using DNA polymorphisms (31).
To date, we have not found a DNA polymorphism in 20 unrelated Caucasians
by hybridizing the pPTHm122 probe to DNA cleaved with the enzymes
EcoRI, BamHI, or HindIII; however, more extensive studies with other
enzymes may reveal polymorphisms useful for linkage studies.

DISCUSSION

These data establish the location of the *PTH* gene on human chromo-
some 11 and suggest that it is localized on the short arm. This region is of
interest since several polymorphic genes are there including insulin (27–30),
β-globin (23–26), and the protooncogene c-Ha-*ras*1 (32). Although our initial
survey did not uncover a polymorphism in *PTH* or its surrounding sequences,
it should be possible to find a DNA polymorphism and use *PTH* in linkage
studies with these markers.

Since there are several inherited forms of abnormal PTH synthesis, i.e.,
hypo- or hyperparathyroidism (33–39), it is of interest to determine if these
diseases are linked to the structural locus for PTH. Thus, a more extensive
search for *PTH* polymorphisms would be warranted. The cloned gene and
associated polymorphisms will be valuable tools for determining if these
lesions are the result of gene mutation and perhaps will provide a means of
diagnosing these inherited diseases.

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Cloning and nucleotide sequence of DNA coding for bovine preproparathyroid hormone

(recombinant DNA/parathyroid hormone/mRNA/restriction map/codon usage)

HENRY M. KRONENBERG*†, BARBARA E. MCDEVITT*†, JOSEPH A. MAJZOUB*†, JEREMY NATHANS†, PHILLIP A. SHARP†‡, JOHN T. POTTS, JR.*, AND ALEXANDER RICH†

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Contributed by Alexander Rich, July 10, 1979

ABSTRACT   We have cloned in Escherichia coli a DNA copy of mRNA coding for bovine preproparathyroid hormone. Double-stranded DNA was inserted into the Pst I site in plasmid pBR322 by using the poly(dG)·poly(dC) homopolymer extension technique to join the DNA molecules. Recombinant plasmids coding for preproparathyroid hormone were identified by the plasmid's ability to arrest specifically the translation of preproparathyroid hormone mRNA. The nucleotide sequence of the largest recombinant was determined by using both chemical and enzymatic techniques. The parathyroid insert contains 470 nucleotides—102 nucleotides from the 5' noncoding region of the mRNA, 345 nucleotides representing the entire coding region, and 23 nucleotides from the 3' noncoding region. The coding sequence clarifies the hormone's amino acid sequence, which has been disputed. Codon usage is discussed.

Parathyroid hormone (PTH) regulates the blood level of calcium, and in turn, blood calcium regulates the secretion of PTH. Although modulators of PTH secretion have been extensively delineated (1), the regulation of synthesis of PTH is poorly understood. Pulse–chase studies of parathyroid gland slices (2) and translation of mRNA in cell-free systems (3) have shown that PTH (84 amino acids) is first synthesized as a 115-amino acid precursor, preproparathyroid hormone (PreproPTH), with a 31-amino acid NH₂-terminal extension. The first 25 amino acids are rapidly cleaved to yield the 90-amino acid proparathyroid hormone, which is subsequently converted to PTH. As a first step in studying further the regulation of PTH biosynthesis, we have cloned in Escherichia coli a DNA copy of PTH mRNA. Nucleotide sequence analysis of the cloned DNA demonstrates that one clone contains the entire coding region plus portions of both the 5' and 3' noncoding regions of the mRNA.

MATERIALS AND METHODS

Enzymes. Reverse transcriptase was provided by J. W. Beard (Life Sciences, St. Petersburg, FL). DNA polymerase I and polynucleotide kinase were purchased from Boehringer Mannheim, S1 nuclease and lysozyme were from Sigma, terminal transferase was from Bethesda Research (Cockeysville, MD), and restriction endonucleases and DNA polymerase I (large fragment) were from New England Biolabs.

Bacteria and Nucleic Acids. E. coli χ1776 (4), a certified EK2 host, was provided by R. Curtiss and E. coli carrying plasmid pBR322 (5) was provided by A. Bothwell. pBR322, a certified EK2 vector,§ after amplification with chloramphenicol was purified on two CsCl₂ gradients after use of the cleared lysate technique (6). PTH mRNA was partially purified as

described (7). Vesicular stomatitis virus RNA was isolated as described (8).

Synthesis of Poly(dC)-Tailed Double-Stranded DNA. DNA complementary in sequence to PTH mRNA was synthesized by using minor modifications of the protocols of Friedman and Rosbash (9) and Efstratiadis et al. (10). mRNA (18 μg) was incubated in a 2.2-ml reaction mixture containing 169 units of reverse transcriptase (220 μl), 50 mM Tris·HCl (pH 8.3), oligo(dT) at 27 μg/ml, 200 μM [α-³²P]dATP and dTTP (both 68 mCi/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels), 900 μM dGTP and dCTP, 120 mM KCl, 10 mM dithiothreitol, and 10 mM MgCl₂. After 1 hr at 43°C the reaction mixture was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), 55 μg of E. coli tRNA was added, and the nucleic acids were precipitated with 2 vol of ethanol. The pellet was treated with NaOH, neutralized, and chromatographed over Sephadex G-100 as described (10).

The cDNA was made double-stranded with DNA polymerase I according to the method of Efstratiadis et al. (11), except that 1/15 vol of a HeLa cell S-100 fraction (12) provided by J. Rose was added to 1 vol of reaction mixture. The nucleic acid was then extracted with phenol/chloroform, precipitated with ethanol, passed over a column of Sephadex G-100, digested with S1 nuclease (11), extracted again with phenol/chloroform, and precipitated with ethanol. The DNA was then centrifuged through a gradient of 5–20% sucrose in 0.8 M NaCl as described (13). The largest molecules (20 ng) were dialyzed against 10 mM Tris·HCl, pH 7.6/0.1 mM Na₂ EDTA, 5 μg of tRNA was added, and the nucleic acids were ethanol-precipitated. Poly(dC) homopolymer extensions, approximately 20 nucleotides long, were added by using terminal transferase as described (14). Similarly, poly(dG) homopolymer extensions, 20 nucleotides long, were added to Pst I-cut pBR322 DNA after phenol extraction, dialysis, and ethanol precipitation of the DNA.

Transfection of E. coli χ1776. Poly(dG)-tailed pBR322 (400 ng) and 30 ng of poly(dC)-tailed parathyroid DNA were annealed in 0.1 M NaCl/10 mM Tris·HCl, pH 7.6/0.1 mM EDTA for 2 min at 65°C and then for 2 hr at 42°C. E. coli χ1776 was grown and treated with 70 mM MgCl₂/30 mM CaCl₂/40 mM Na acetate, pH 5.6, as described (15). Transformation was performed in a biological safety cabinet in a P3 physical containment facility according to the then current National Institutes of Health guidelines for recombinant DNA research.§

Hybrid-Arrested Translation. Individual clones were grown in 200 ml of FB medium (25 g of tryptone/7.5 g of yeast extract/1 g of glucose/6 g of NaCl/50 mM Tris·HCl, pH 7.6, taken to 1 liter) supplemented with thymidine 50 at μg/ml,

Abbreviations: PTH, parathyroid hormone; PreproPTH, preproparathyroid hormone
§ National Institutes of Health, Federal Register, July 7, 1976.

diaminopimelic acid at 100 µg/ml, and tetracycline at 20 µg/ml; then, they were amplified with chloramphenicol. The plasmid DNA was isolated by using the cleared lysate technique (6) followed by two centrifugations in CsCl/ethidium bromide equilibrium gradients. Hybrid-arrested translation was performed as described (16).

Restriction-Enzyme Analysis. Five micrograms of the PTH recombinant plasmid was digested with *Pst* I in a 100-µl reaction mixture. After digestion, 5 units of DNA polymerase I (large fragment) was added and incubation was continued for 5 min at 37°C. The reaction mixture was then transferred to another tube containing 10 µCi of each of four [α-^{32}P]dNTPs (400 Ci/mmol) and the reaction was continued for 5 min at 37°C. Then the reaction mixture was adjusted to 40 µM of each of the four dNTPs and the reaction was continued for 10 min. The digest, now possessing molecules labeled with ^{32}P at the 3' end, was precipitated with ethanol and then fractionated by electrophoresis on a 10% polyacrylamide slab gel in Tris/borate/EDTA buffer (17). The eukaryotic insert was then eluted from the gel (18). Aliquots of the insert were then digested with restriction enzymes and the procedures of 3'-end labeling and gel electrophoresis were repeated. For reactions involving enzymes that produce DNA with single-stranded 5' extensions (e.g., *Hin*fI), the enzyme was removed by chloroform extraction before end-labeling.

DNA Sequence Analysis. Both the chemical (18) and enzymatic dideoxynucleotide (19) methods were used without modification. Restriction fragments were 5'-end labeled by using the polynucleotide kinase exchange reaction (20).

RESULTS

Construction of cDNA Clones and Translational Analysis. Earlier studies (7, 21) have established that the mRNA coding for PreproPTH is a predominant mRNA in the parathyroid gland and that use of formamide/sucrose gradients and oligo(dT)-cellulose chromatography can lead to partial purification of the mRNA. Furthermore, cDNA made from this mRNA can function in a linked transcription/translation system to produce the entire PreproPTH molecule (7) and thus contains all the structural information encoded in the mRNA. We made this cDNA double stranded, selected the largest DNA molecule on a sucrose gradient, and introduced the DNA into plasmid pBR322 by using the poly(dG)·poly(dC) homopolymer extension technique. From 15 ng of double-stranded parathyroid DNA, we obtained 49 transformed colonies with the pattern of tetracycline resistance and ampicillin sensitivity, the pattern expected from bacteria containing pBR322 with eukaryotic inserts at the *Pst* I site.

Six of these clones were grown and the plasmid DNA was isolated. We found it important to purify the plasmid DNA by two cycles of centrifugation through CsCl, because DNA after only one centrifugation gave erratic results in hybrid-arrested translation. After *Pst* I digestion, phenol extraction, and ethanol precipitation, each DNA, as well as control pBR322 DNA, was hybridized to 50 ng of parathyroid gland mRNA as indicated in Fig. 1. Vesicular stomatitis virus RNA was added to the mixture to provide control mRNAs that stimulated protein synthesis as well as did PreproPTH mRNA. After annealing,
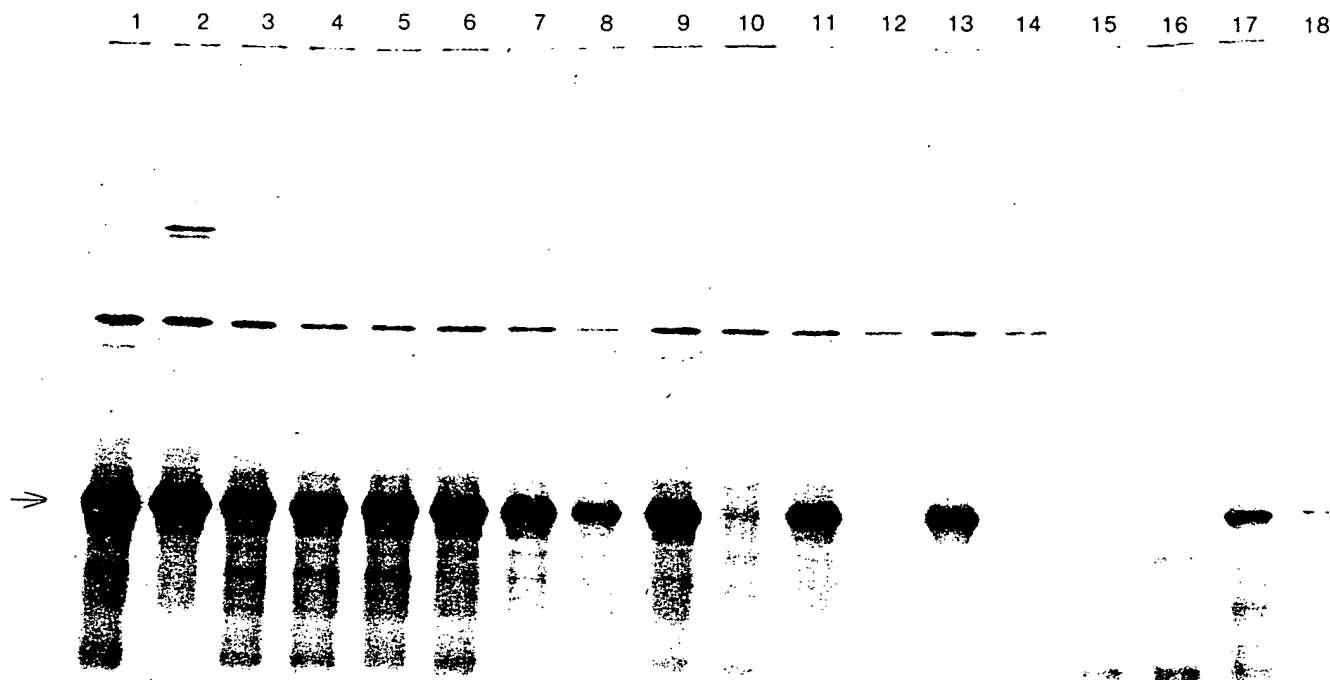
FIG. 1.   Hybrid-arrested translation. Plasmid DNA was annealed with 50 ng of parathyroid gland mRNA that had been purified by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation. A similar amount of VSV mRNA was added to reactions 1–16. One half of each sample was heated and cooled quickly after annealing. After ethanol precipitation along with 25 µg of yeast tRNA, each sample was added to a 50-µl wheat germ translation reaction mixture in the presence of [^{35}S]methionine. Five microliters of each sample was then electrophoresed through a 15–20% polyacrylamide gradient gel containing sodium dodecyl sulfate, and fluorography was performed on the dried gel. Arrow points to PreproPTH. Lanes: 1, no DNA was added and mRNA was mock-annealed, heated, and quick-cooled; 2, same as lane 1 but with no heating; 3, 1 µg of pBR322 DNA, heated; 4, same as lane 3 but with no heating; 5, 2 µg of pBR322 DNA, heated; 6, same as lane 5 but with no heating; 7, 4 µg of pBR322 DNA, heated; 8, same as lane 7 but with no heating; 9, 1 µg of pPTHm1 DNA, heated; 10, same as lane 9 but with no heating; 11, 2 µg of pPTHm1 DNA, heated; 12, same as lane 11 but with no heating; 13, no DNA or mRNA added, heated; 14, same as lane 13 but with no heating; 15, no DNA added, mRNA was not mock-annealed but was heated and added directly to translation mixture; 16, same as lane 15 but with no heating; 17, PTH mRNA mock-annealed without DNA or VSV mRNA; 18, same as lane 17 but with no heating.

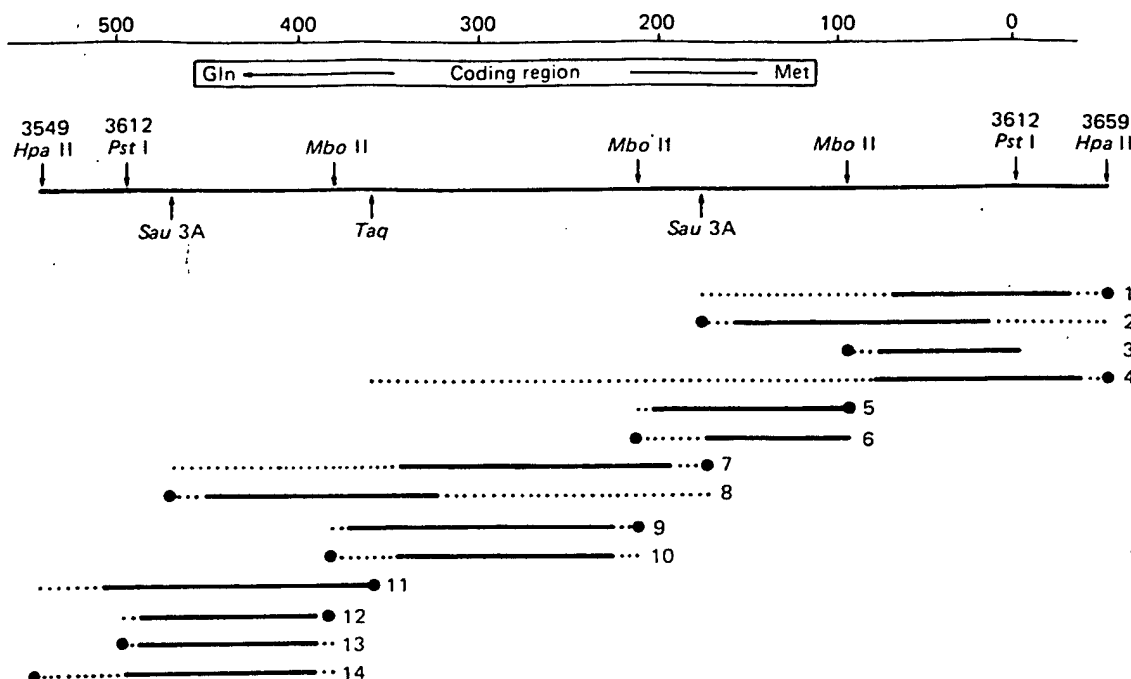FIG. 2.　Restriction enzyme map of pPTHm1. Numbers above the *Hpa* II and *Pst* I sites correspond to the cutting sites on pBR322 (32); numbers above the insert correspond to the numbers used in Fig. 3 to describe the eukaryotic insert. Lines below the map indicate the restriction fragments used in chemical sequence determinations. Solid parts of each line indicate areas of readable sequence. Fragments 5, 6, 9, and 10 were separated on a 5% polyacrylamide strand-separating gel (18); all other fragments were generated by using combinations of restriction enzyme digestions to produce molecules with only one 5'-labeled end. Dots represent the 5' end so labeled. *Mbo* II-generated fragments 5, 6, 9, and 10 were also digested with exonuclease III and used as primers for dideoxynucleotide sequence analysis, using as template pPTHm1 DNA cut with *Eco*RI, and then were partially digested with exonuclease III or T7 exonuclease.

the DNA/RNA mixture was divided in half, and one half was heated and cooled quickly to break up the hybrids. After precipitation with ethanol, the nucleic acids were then added to a wheat germ cell-free protein-synthesizing system in the presence of [^{35}S]methionine. The proteins made were then electrophoresed, and autoradiography was performed. Fig. 1 shows that pPTHm1 DNA, in each of three concentrations, inhibited the translation of PreproPTH while not affecting the
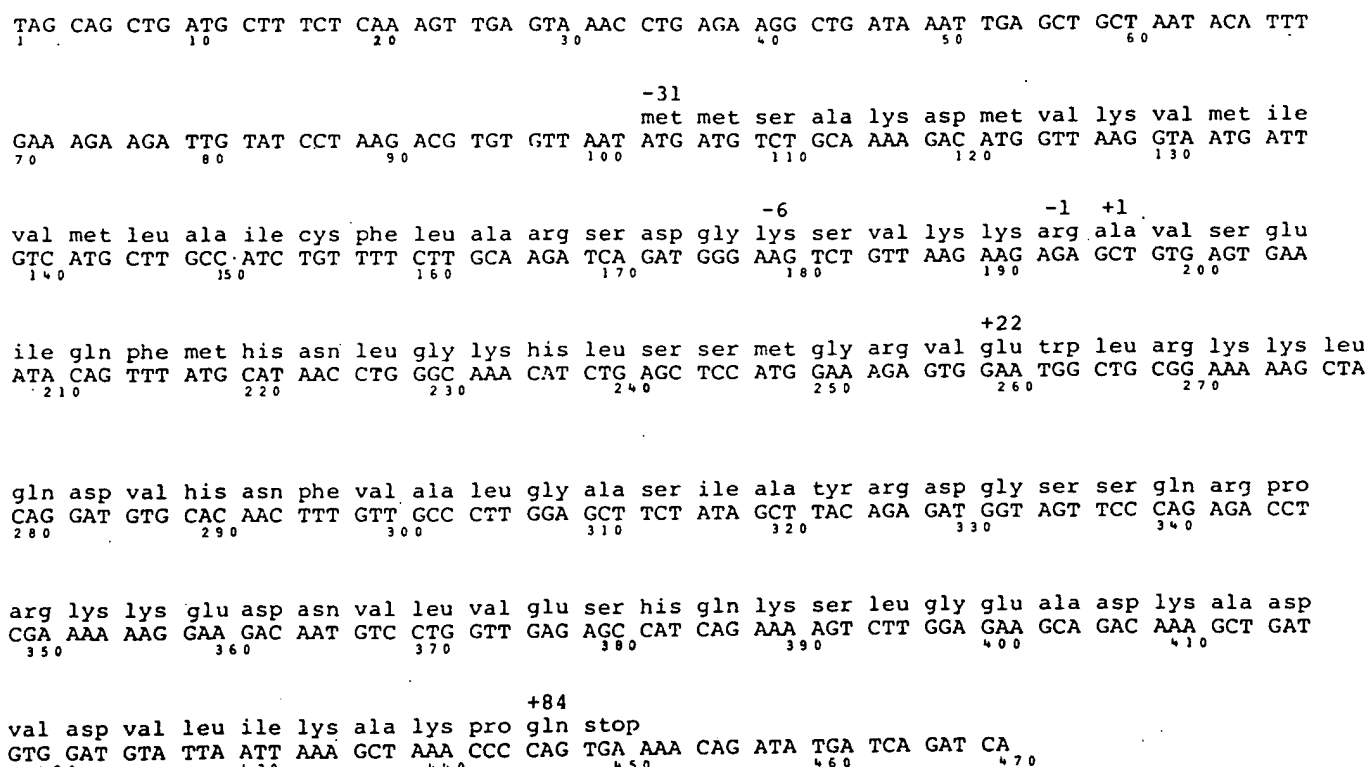

FIG. 3.　Nucleotide sequence of sense strand of parathyroid insert in pPTHm1. Orientation of insert is indicated in Fig. 2. Numbers above the protein sequence refer to amino acid position; by convention −31 to −7 is "pre" sequence, −6 to −1 is "pro" sequence, and +1 to +84 is PTH sequence.

translation of other proteins (lanes 10, 12, and 14). Furthermore, heating and quick-cooling of the nucleic acid restored the translation of PreproPTH. In contrast, pBR322 DNA at similar concentrations had no effect on protein synthesis (lanes 3–8).

By using DNA from pPTHml as a probe, we screened our other 48 clones for PTH inserts larger than that of pPTHml. A toothpick tip was used to mix a small portion of each individual colony taken from an agar plate in Tris/EDTA/sucrose/lysozyme as described (22). After precipitation of proteins with potassium acetate and sodium dodecyl sulfate and digestion of the nucleic acids with RNase and then *Pst* I, DNA from each colony was electrophoresed on a 1.4% agarose gel (23) and transferred to nitrocellulose (24). PTH inserts with intact *Pst* I sites were detected by hybridization with nick-translated pPTHml DNA. Seven of 48 colonies yielded bands of size similar to the insert in pPTHml, but no larger bands were found (data not shown).

Restriction Enzyme Analysis and DNA Sequence Analysis. The eukaryotic insert of pPTHml was isolated, digested with a series of restriction endonucleases, labeled with ^{32}P at the 3′ end, and fractionated by electrophoresis on a 10% acrylamide gel (17). The enzymes *Bam*HI, *Hin*dIII, *Eco*RI, *Sal* I, *Hin*cII, *Hin*fI, *Hae* III, *Hpa* I, *Bgl* II, *Xho* I, *Hha* I, and *Msp* I did not cut this insert. *Mbo* II, *Alu* I, *Taq* I, and *Sau* 3A did cut the insert (Fig. 2). (*Alu* sites are omitted from the figure because fragments cut by *Alu* I were not used in the subsequent sequence analyses.)

The sequences of the restriction enzyme-generated fragments noted in the legend to Fig. 2 were determined by the chemical method. Furthermore, the *Mbo* II fragments were used as primers for dideoxynucleotide sequence analysis after either partial exonuclease III or T7 exonuclease digestion of *Eco*RI-cut pPTHml DNA.

We found that the 5′-end labeling of restriction fragments generated by the enzymes *Mbo* II and *Pst* I was inefficient, presumably because these fragments lacked 5′ single-stranded DNA extensions. Therefore, we added DNA polymerase (large fragment), 1 unit/μg of DNA, for 5 min at 37°C to the restriction enzyme digestion reaction mixtures at the end of the digestions and then extracted the DNA with phenol to terminate the reactions. These modified fragments were more efficiently end-labeled, presumably because the 3′ → 5′ exonuclease activity of the DNA polymerase I-generated 5′ single-stranded DNA extensions at the ends of the fragments.

Because we found that the nucleotides at the beginnings and ends of restriction fragments were usually difficult to resolve, we used a number of different restriction enzymes to generate overlapping fragments for analysis by chemical sequence analysis. Furthermore, the use of the dideoxynucleotide enzymatic sequence analysis strategy confirmed the sequence derived by the chemical approach. Because restriction fragments are used as primers of DNA synthesis in this technique, the DNA synthesized by using specific primers allowed additional independent data for ordering the sequence-analyzed fragments. Fig. 3 depicts the sequence thus established.

DISCUSSION

The parathyroid DNA in pPTHml, 470 nucleotides in length, represents about 70% of the nucleotide sequence of PreproPTH mRNA, as estimated from the size of the mRNA determined by electrophoresis through 98% formamide/polyacrylamide gels (31). The sequence includes the entire coding region of the mRNA, 102 residues of the 5′ noncoding region, and 23 residues of the 3′ noncoding region. The sequence of pPTHml DNA confirms the amino acid sequence of bovine PreproPTH, as determined by a combination of traditional (25, 34) and ra-

diomicrosequence analysis (3) approaches. Furthermore, the GAA codon coding for the glutamic acid at position 22, found when the sequences of DNA fragments 7, 8, and 9 were determined (see Fig. 2), supports our previous assignment, a finding that has been disputed by another group (25). Bovine genetic heterogeneity could still, of course, explain the disparate sequences found at position 22. We cannot, in addition, exclude the possibility that the sequence was modified by the process of cloning, but the accurate prediction of the rest of the PreproPTH amino acid sequence is reassuring, as are other examples of faithful cDNA cloning (26, 30). To minimize chances of error in determination of nucleotide sequences, every nucleotide in the coding sequence was ascertained at least twice (see Fig. 2).

Codon usage analysis (Table 1) demonstrates some uneven usage of codons, but, as noted by others (26, 30), comparisons with usage patterns in other eukaryotic mRNA show no consistent patterns. For instance, when one compares the codon usage patterns between bovine corticotropin-β-lipotropin precursor mRNA and bovine PreproPTH mRNA, the codons UUC, CUC, and AGG, unused in PreproPTH mRNA, are frequently used in corticotropin-β-lipotropin precursor mRNA. The glutamine codon CAA is unused in PreproPTH mRNA and used only 1 of 10 possible times in corticotropin-β-lipotropin precursor mRNA. Also, as noted by others (30), the dinucleotide CG is used infrequently—two times—in the coding region. In marked contrast to the pattern in several other mammalian sequences (26, 30), however, no preference is found for G or C in the third codon position—30% of codons terminate in T, 17% terminate in C, 27% terminate in A, and 26% terminate in G.

Table 1. Codon usage in bovine PreproPTH mRNA

Amino acid	Codon	Usage	Amino acid	Codon	Usage
Phe	UUU	3	Tyr	UAU	0
	UUC	0		UAC	1
Leu	UUA	1	Term	UAA	0
	UUG	0	Term	UAG	0
	CUU	4	His	CAU	3
	CUC	0		CAC	1
	CUA	1	Gln	CAA	0
	CUG	4		CAG	5
Ile	AUU	2	Asn	AAU	1
	AUC	1		AAC	2
	AUA	2	Lys	AAA	8
Met	AUG	7		AAG	6
Val	GUU	4	Asp	GAU	5
	GUC	2		GAC	3
	CUA	2	Glu	GAA	5
	GUG	4		GAG	1
Ser	UCU	3	Cys	UGU	1
	UCC	2		UGC	0
	UCA	1	Term	UGA	1
Pro	UCG	0	Trp	UGG	1
	CCU	1	Arg	CGU	0
	CCC	1		CGC	0
Thr	CCA	0		CGA	1
	CCG	0		CGG	1
	ACU	0	Ser	AGU	3
Ala	ACC	0		AGC	2
	ACC	0	Arg	AGA	5
	ACG	0		AGG	0
	GCU	4	Gly	GGU	1
	GCC	2		GGC	1
	GCA	3		GGA	2
	GCG	1		GGG	1

Based on a statistical analysis of peptide sequences, Cohn *et al.* (27) have proposed that PreproPTH originated after gene duplication. They noted nine amino acid matches when peptides −27 to +22 and +26 to +74 were aligned. They calculated $P = 0.0001$ for this being a chance occurrence. When the nucleotide sequences of these regions are similarly aligned, 53 of 147 nucleotides match. Thus, the nucleotide sequence, like the amino acid sequence, suggests similarities that are unlikely to have occurred by chance. Similar comparisons have been made between the sequences of rat growth hormone and human chorionic somatomammotropic hormone (30) and between four regions of the bovine corticotropin-β-lipotropin precursor (26). In both of these comparisons, there was little variation in the third nucleotide of triplets, even though that variation would not have resulted in a changed amino acid sequence. In contrast, in the PreproPTH comparison, only two of nine third bases remain invariant when the triplets coding for the nine invariant amino acids are compared—no more than expected by chance. Furthermore, when one excludes the nucleotides associated with the amino acid matches, only 32 of 120 nucleotides match, again no more than expected by chance. Thus, the mRNA sequence, although consistent with the hypothesis that the gene arose by duplication, does not add evidence beyond that provided already by the amino acid homologies. Perhaps the pattern of intervening sequences that may exist in the genomic DNA will shed light on the origin of the PreproPTH sequence. We might expect to find, for instance, an intervening sequence separating the duplicated structural regions if RNA splicing acts as a means of bringing together in one mRNA two sets of sequences that arose by tandem gene duplication.

The AUG at position 10–13 in the 5′ noncoding region is only the second example in eukaryotes of an AUG in the 5′ noncoding region of an mRNA, the first example being the mRNA coding for VP1 of simian virus 40. The sequences of all other mRNAs are consistent with the hypothesis of Kozak that ribosomes bind at a 5′-terminal cap and scan the mRNA until they initiate at the first available AUG (28). Kozak notes that the two AUGs in the 5′ noncoding region of VP1 mRNA may well be part of a 5′-terminal double-stranded RNA stem and therefore might be sequestered from the ribosome. It is of interest, then, that the AUG in the PreproPTH sequence may be part of a small interior loop in an extensive stem structure involving nucleotides 1–20 and 34–51 [$\Delta G = -15.6$ kcal, including eight consecutive nucleotide pairs at the base of the stem (29)]. Alternatively, the AUG at position 10–13 might serve as an initiator codon for a pentapeptide terminated by UGA at position 25–27.

The sequence AUCCU at position 83–87 may bind to the sequence UAGGA close to the 3′ end of 18S ribosomal RNA. Hagenbüchle and coworkers (33) have pointed out that most eukaryotic mRNAs contain sequences that could bind to this highly conserved purine-rich sequence in 18S RNA, thus favoring initiation of protein synthesis.

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(54) **Process and system for producing biological materials from encapsulated cells**

(57) Cells (10) which produce a substance of interest are encapsulated within semipermeable membranes (12) having an upper limit of permeability sufficient to allow traverse of ions, amino acids and other cell nutrients (20) and then suspended in a culture medium containing these nutrients. Serum components (18) or other high molecular weight materials needed for ongoing viability and normal *in vitro* metabolism of certain types of cells may be included within the intracapsular volume and may be excluded from the extracapsular medium by limiting the permeability of the membranes (12). The substance of interest (22) to be harvested collects either in the intracapsular volume or the extracapsular medium, depending on the degree of permeability of the membranes and the molecular size of the substance.
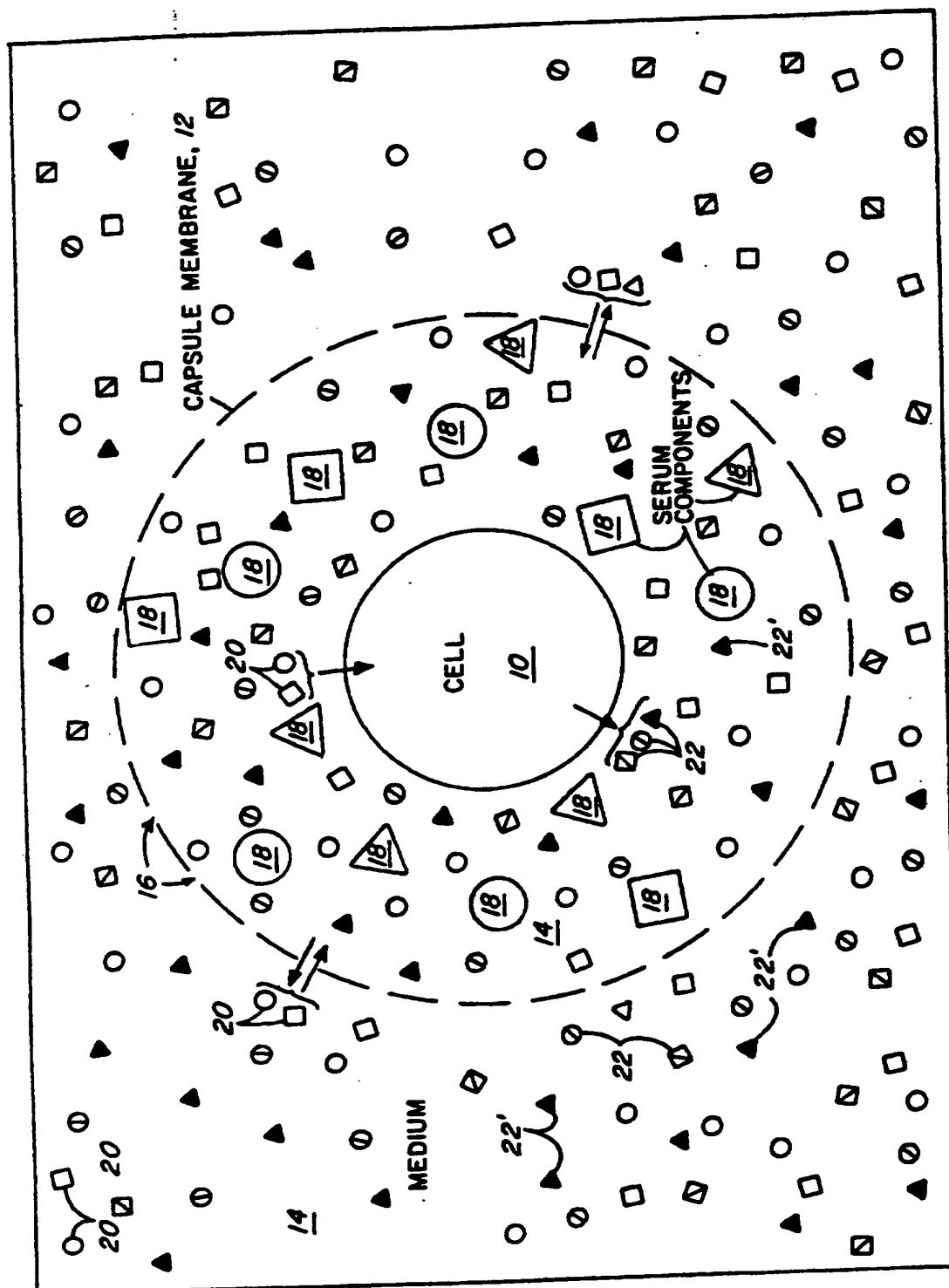
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FIG. 1

CAPSULE MEMBRANE, 12

SERUM COMPONENTS

CELL
10

MEDIUM

▲— SUBSTANCE OF INTEREST PRODUCED BY CELL

O□-LOW MOLECULAR WEIGHT SUBSTANCES INGESTED BY CELL

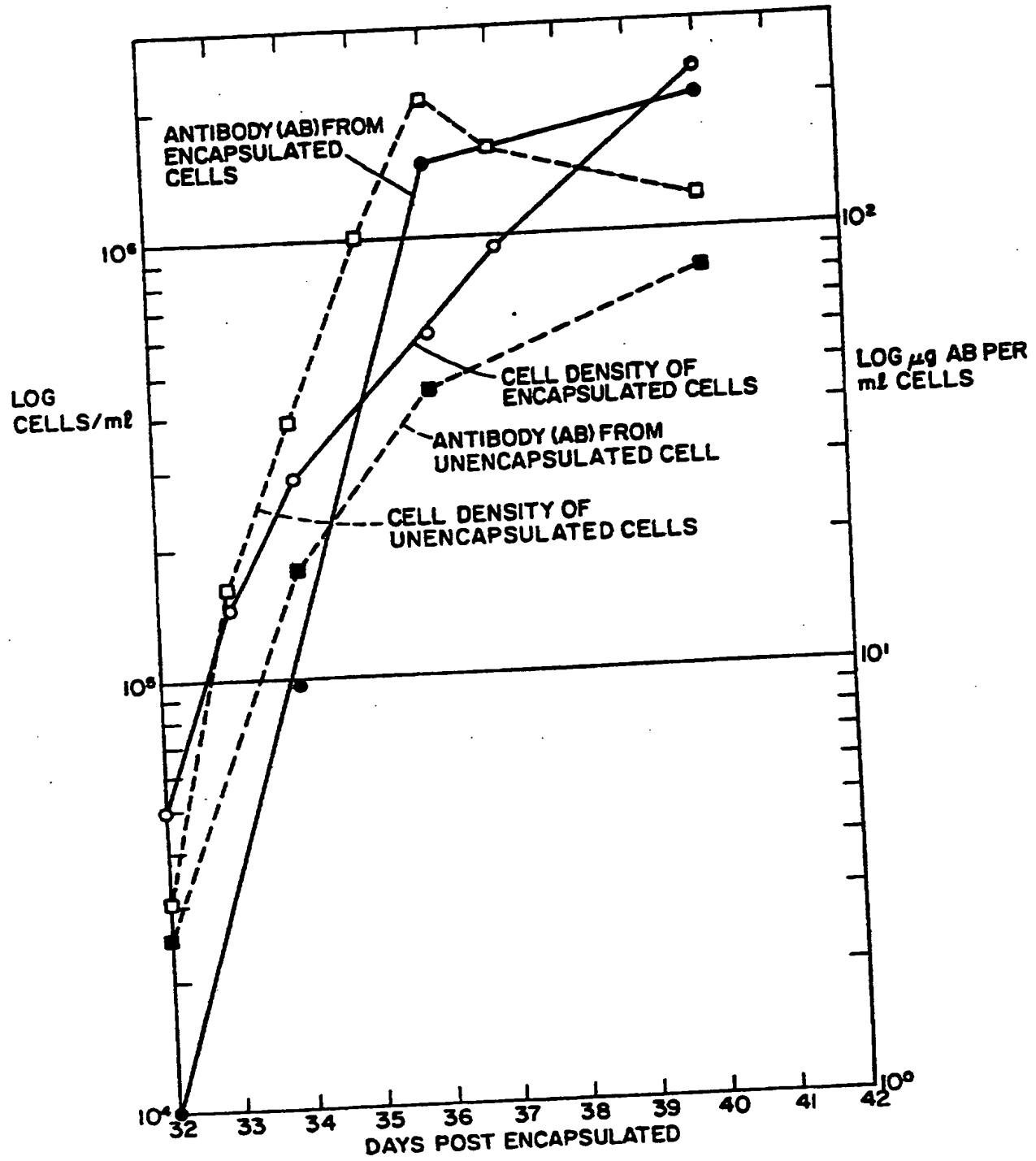⊘▨-EXTRANEOUS METABOLIC PRODUCTS

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FIG. 2

SPECIFICATION
Process and system for producing biological
materials

This invention relates to a method of and
5 system for producing biological materials by cells.
Advances in cellular biology have shown that
the cells of various higher organisms produce
small quantities of substances having significant
potential or demonstrable utility for the treatment
10 or diagnosis of disease. Examples of such
substances abound in the literature and include
various biological response modifiers such as
hormones, interferons, and lymphokines, as well
as other substances such as antibodies used in
15 diagnostic testing. Cell cultures of microbial origin
have long been used to produce large quantities of
antibiotics.
Especially in cell cultures derived from higher
animals, there is an ever present danger of
20 bacterial or other contamination. Also, in most
instances the quantities of the substance of
interest produced by cell cultures are very small
and the substance often collects in the culture
medium, which contains a complex mixture of
25 serum proteins and other substances. This makes
isolation and purification of the substance of
interest difficult.
This invention aims to provide an improved
process and system for producing biological
30 materials such as antibodies and biological
response modifiers such as hormones, interferons
and lymphokines from encapsulated viable cells.
The encapsulation aims to maintain the cells in a
healthful microenvironment and to separate
35 products of cell metabolism from high molecular
weight materials needed for viability and
maintenance of the cells.
According to the present invention, there is
provided a process for producing a substance
40 which is produced by living cells, wherein the
process comprises the steps of:
A. encapsulating the cells within membranes
having a selected upper limit of permeability;
B. suspending the encapsulated cells in an
45 aqueous culture medium;
C. allowing said cells to undergo metabolism in
vitro and to secrete the required substance; and
D. harvesting the substance either from the
aqueous culture medium or from within the
50 membranes.
The invention also provides a system for
culturing living cells comprising encapsulated
viable cells suspended in an aqueous culture
medium, membranes encapsulating the cells
55 being characterized by an upper limit of
permeability sufficient to allow traverse of
nutrients required by the cells, the membranes
enclosing suspended viable cells disposed in a
medium which includes all components needed to
60 maintain viability of said cells which are of a size
beyond the upper permeability limit of the
membranes, and the aqueous culture medium
comprising all components needed to maintain
viability of said cells which have a molecular size

65 below the upper permeability limit.
This invention provides a system and process
for producing substances by use of living cells. The
practice of the invention has the inherent dual
advantages of providing a protective environment
70 for the cells of the culture system and providing a
means of collecting substances of interest in a
medium having fewer admixed extraneous
components. The invention may be used to
separate the substance of interest from higher
75 molecular weight serum proteins and the like
normally required to support the ongoing viability
and metabolism of the producing cells.
Alternatively, the invention may be used to collect
the substance of interest in a medium containing
80 relatively small quantities of low molecular weight
nutrients or cell metabolic products.
The process comprises the steps of
encapsulating cells within a membrane which is
permeable to the nutrients, ions, and other
85 relatively low molecular weight materials needed
for normal metabolism and ongoing viability of the
cells. The membrane may or may not be
permeable to the substance of interest secreted by
the cells, but in any case will have an upper limit
90 of permeability sufficient to allow traverse of some
molecules having a molecular weight of some
selected level generally below about 2.0×10^5
daltons, e.g. 1.5×10^5. The capsules so produced
are suspended in a conventional aqueous culture
95 medium, and the encapsulated cells are allowed to
undergo normal in vitro metabolism. Substances
of a molecular weight below the upper
permeability limit of the membrane which are
secreted by the cells permeate the membrane and
100 collect in the culture medium. Advantageously,
high molecular weight substances such as serum
proteins which are required for health and viability
of many types of cell cultures from higher animals,
but which typically are themselves not consumed,
105 may be included in the microcapsules where they
are confined and prevented from diffusing into the
culture medium. Substances which the cell culture
consumes during metabolism having a molecular
weight low enough to permit diffusion through the
110 capsule membranes pass therethrough from the
culture medium. Metabolic products of the cells
having molecular dimensions sufficiently small to
allow passage through the membrane diffuse into
the medium external to the capsules. The
115 substances of interest, if of a molecular weight
below the upper limit of permeability, diffuse into
the extracapsular medium where they can be
harvested relatively easily because of the absence
of contaminating higher molecular weight
120 materials present in prior art unencapsulated cell
cultures. If the substance of interest has a
molecular weight in excess of the upper limit of
permeability of the membranes, then it collects in
the capsules which may subsequently be isolated
125 from the medium and disrupted for recovery
procedures.
The invention is essentially unlimited with
respect to the types of cells which may be
included within the capsule membranes.

Specifically, it is contemplated that cultures of cells from the tissue of all higher animals as well as micro-organisms may be employed. Fused cells, e.g., hybridoma cells, or genetically modified
5 cells produced, for example, by the emerging recombinant DNA technology, can likewise be encapsulated without difficulty. In short, provided there exists a culture medium operable to maintain *in vitro* the cell type in question, that cell
10 type can be utilized in accordance with the techniques disclosed herein. Non-limiting examples of the types of substances that may be produced in accordance with the process and by the system of the invention include insulin,
15 glycogen, prolactin, somatostatin, thyroxin, steroid hormones, pituitary hormones, interferons, follicle-stimulating hormones (FSH), PTH, and antibodies.

The system of the invention comprises encapsulated viable cells suspended in an
20 aqueous culture medium. The cells are encapsulated within membranes characterized by an upper limit of permeability sufficient to allow traverse of the nutrients needed for cell metabolism and ongoing viability. The membranes
25 enclose both the cells and a medium which includes all components needed to maintain metabolism of the cells and which are of a size range in excess of the upper permeability limit of the membranes. The culture medium comprises
30 components needed to maintain viability of the cells and which have a molecular weight below the upper permeability limit of the membranes.

The invention will now be described in more detail by way of example with reference to the
35 accompanying drawings, in which:

Figure 1 is a schematic diagram illustrating the concept of the invention, and

Figure 2 is a graph showing the results of an experiment described in Example 5 hereafter.
40 The broad concept of the invention is to interpose a semipermeable membrane about individual cells or groups of cells so as to provide a microenvironment therefor complete with the cell culture medium and separated by the membrane
45 from an external aqueous medium. Cells of mammalian origin typically require for ongoing health and viability the presence of serum proteins, a portion of which have a molecular weight in excess of about 65,000—150,000
50 daltons.

In the prior art technique of unencapsulated cell culturing, materials of interest secreted from the cells are dispersed in the culture medium and mixed with both high and low molecular weight
55 components. Since the quantities of cell-produced products are typically rather small, isolation of the substance of interest becomes an arduous purification task. Furthermore, mammalian cell cultures are notoriously sensitive to contamination
60 by bacterial or other sources. This necessitates that culturing be conducted using various techniques to maintain sterility and often that antibiotics be included in the medium.

According to the practice of this invention, the
65 foregoing difficulties are alleviated by encapsulating the cells of the culture within semipermeable membranes having a selected limit of permeability generally no greater than about 200,000 daltons, that is, the membrane
70 contains pores which allow substances having a maximum molecular weight at or below the upper permeability limit to traverse the membrane whereas substances of molecular weight above the upper permeability limit are precluded from
75 traversing the membrane. This allows one to encapsulate cells together with a culture medium containing all components needed for ongoing viability, metabolism, and even mitosis, and then to suspend the so-encapsulated cells in a culture
80 medium which contains lower molecular weight substances consumed by the cells but which need not include the required high molecular weight substances.

Typically, cells from higher organisms do not
85 ingest high molecular weight serum proteins and the like, but rather require them in close proximity for ongoing normal biological responses. Salts, amino acids and other lower molecular weight factors which are ingested or metabolized by the
90 cells pass freely through the membrane and may be replenished as needed by simple change of the culture medium external to the capsules. Secreted products of cell metabolism having a molecular weight below the upper limit of membrane
95 permeability collect in the extracapsular medium, where, because of the absence in the medium of the high molecular weight materials, harvesting and isolation of the metabolic products of interest are simplified. Harvesting of products of
100 interest having a molecular weight above the upper permeability limit is also aided in that such products collect within the capsules and are not dispersed in the extracapsular volume.

The concept of the invention, as applied to
105 lower molecular weight cell products, is schematically illustrated in the drawing. As shown, a cell 10 is disposed within a capsule membrane 12 having pores 16. High molecular weight factors 18 are enclosed within membrane
110 12 and are free to circulate within the confines of the membrane in the medium 14. Components 20 needed by the cell as well as metabolic products 22 including the substance of interest 22' freely circulate in both the intracapsular and
115 extracapsular media and traverse the membrane through pores 16. As required on a periodic (or continuous) basis, the extracapsular medium together with all of its components can be separated by aspiration or the like from the
120 capsules themselves and replaced with fresh medium. The collected medium will be substantially free of high molecular weight components 18, thus simplifying the harvesting and isolation procedures. Furthermore, the cell 10
125 remains protected within the intracapsular microenvironment at all times.

In some cases, e.g., in order to stimulate production by encapsulated cells of a particular substance of interest, it is required to subject the
130 cells to high molecular weight components having

molecular dimensions too large to traverse the
membrane. An example is the production of
interferon from human fibroblasts, leukocytes, or
lymphoblastoid cells which are induced to secrete
5 interferon by treatment with certain viruses or
high molecular weight nucleic acids. In such a
case, if the upper permeability limit of the
membranes is less than the molecular weight of
the inducing factor, the cells must be subjected to
10 interferon induction prior to encapsulation, or the
capsule membranes, after culture of the cells,
must be selectively disrupted to allow such high
molecular weight materials to be ingested by the
cell. Our copending application 82 filed on the
15 same day as this application, and naming Franklin
Lim as the sole inventor, discloses a method of
selectively disrupting certain types of capsule
membranes which may be used for these and
other purposes without damage to the cells.
20   The process of the invention depends on one's
ability to form semipermeable membranes about
cells without simultaneously adversely affecting
their ongoing viability. One suitable encapsulation
process is set forth in detail below.

25 Cell Encapsulation
      The tissue or cells to be encapsulated are
suspended in an aqueous medium suitable for
maintenance or for supporting the ongoing
metabolic processes of the particular tissue or cell
30 type involved. Media suitable for this purpose are
available commercially. The average diameter of
the material to be encapsulated can vary widely
between a few micrometers to several millimeters.
However, best results are achieved with capsules
35 of a size in the range of 300—1000 micrometers.
Mammalian islets of Langerhans are typically 50
to 200 micrometers in diameter. Individual cells
such as fibroblasts, leukocytes, lymphoblastoids,
pancreatic beta cells, alpha cells, delta cells,
40 various ratios thereof, or other tissue units may be
encapsulated as desired. Also microorganisms
may be encapsulated including those which have
been genetically modified by recombinant DNA or
other techniques.
45   The ongoing viability of such living matter is
dependent, inter alia, on the availability of required
nutrients, oxygen transfer, absence of toxic
substances in the medium, and the pH of the
medium. Until recently, it has not been possible to
50 maintain such living matter in a physiologically
compatible environment while simultaneously
encapsulating. The problem has been that the
conditions required for membrane formation have
been lethal or harmful to the tissue, and prior to
55 the invention disclosed in our U.K. patent
application No. 8008971, publication No. GB
2 046 209A, no method of membrane formation
which allowed tissue to survive in a healthy state
had been forthcoming.
60   However, it has been discovered that certain
water-soluble substances which are
physiologically compatible with living tissue and
can be rendered water-insoluble to form a shape-
retaining, coherent mass, can be used to form a

65 "temporary capsule" or protective barrier layer
about individual cells or groups of cells and that
this temporary capsule can be treated to deposit a
more permanent semipermeable membrane about
the cells without damage to the cells. Such a
70 substance is added, typically at a concentration on
the order of a few weight percent, to the tissue
culture medium, which also contains cells of the
culture, serum components (if required) and
optionally, a cellular substrate such as collagen or
75 another high molecular weight, water dispersible
material which acts as an anchoring substrate.
When using collagen, the concentration should be
within the range of about 10 μg/ml to about
1 mg/ml, but preferably on the order of
80 100—500 μg/ml.
      The solution is then formed into droplets
containing tissue together with its medium and is
immediately rendered water-insoluble and gelled,
at least in a surface layer. Thereafter, the shape-
85 retaining temporary capsules are provided with a
more permanent membrane which may itself
subsequently be selectively disrupted if it is
desired to release the tissue without damage.
Where the material used to form the temporary
90 capsules permits, the capsule interior may be
reliquefied after formation of the permanent
membrane. This is done by re-establishing the
conditions in the medium at which the material is
soluble.
95   The material used to form the temporary
capsules may be any non-toxic, water-soluble
material which, by a change in ionic environment
or concentration, can be converted to a shape-
retaining mass. The material should also contain
100 plural, easily ionized anionic moieties, e.g.,
carboxyl groups, which can react by salt formation
with polymers containing plural cationic groups.
As will be explained below, use of this type of
material enables one to deposit a permanent
105 membrane of a selected upper limit of
permeability without difficulty in surface layers of
the temporary capsule.
      The presently preferred materials for forming
the temporary capsule are acidic, water-soluble,
110 natural or synthetic polysaccharide gums. Such
materials are commercially available. They are
typically extracted from vegetable matter and are
often used as additives to various foods. Sodium
alginate is the presently preferred water-soluble
115 gum. Alginate in the molecular weight range of
150,000+ daltons may be used, but because of its
molecular dimensions and viscosity will usually be
unable to permeate the finally formed capsule
membranes. Lower molecular weight alginate,
120 e.g., 50,000—80,000 daltons, is more easily
removed from the intracapsular volume by
diffusion through a membrane of sufficient
porosity and is therefore preferred. Other usable
gums include acidic fractions of guar gum,
125 carageenan, pectin, tragacanth gum, or xanthan
gum.
      These materials comprise glycoside-linked
saccharide chains. Their free acid groups are often
present in the alkali metal ion form, e.g., sodium

form. If a multivalent ion such as calcium or aluminum is exchanged for the alkali metal ion, the water-soluble polysaccharide molecules are "cross-linked" to form a water-insoluble, shape-
5 retaining gel which can be resolubilized on removal of the ions by ion exchange or via a sequestering agent. While essentially any multivalent ion which can form a salt with the acidic gum is operable, it is preferred that
10 physiologically compatible ions, e.g., calcium, be employed. This tends to preserve the tissue in the living state. Other multivalent cations can be used, but it should be noted that magnesium ions are ineffective in gelling sodium alginate.
15   A typical solution composition comprises equal volumes of a cell culture in its medium and a one or two percent solution of gum in physiological saline. When employing sodium alginate, a 1.2 to 1.6 percent solution has been used with success.
20 If the cells to be encapsulated are of the type which require attachment to an anchoring substrate to undergo mitosis, and if the cells are to be grown with the capsules, then collagen or another high molecular weight water-dispersible
25 protein or polypeptide, either natural or synthetic, may be included in the cell culture, and will be confined within the intracapsular volume of the finally formed capsules. If a polymer having plural cationic groups, e.g., polylysine, is employed for
30 this purpose, the cationic groups react with anionic sites on the water-soluble gum to form a substantially water-insoluble matrix intertwined with the gum. Preferred concentrations for such materials are on the order of 100—500 μg/ml of
35 suspension (including gum solution).
    In the next step of the encapsulation process, the gum solution containing the tissue is formed into droplets of a desired size. Thereafter, the droplets are immediately gelled to form shape-
40 retaining masses preferably but not necessarily in spherical or spheroidal form. The drop formations may be conducted by known methods. An exemplary procedure follows.
    A tube containing an aqueous solution of
45 multivalent cations, e.g., 1.5% $CaCl_2$ solution, is fitted with a stopper which holds a drop forming apparatus. The apparatus consists of a housing having an upper air intake nozzle and an elongate hollow body friction fitted into the stopper. A
50 10 cc syringe equipped with a stepping pump is mounted atop the housing with, e.g., a 0.01 inch I.D. (0.25 mm) Teflon coated needle passing through the length of the housing. The interior of the housing is designed such that the tip of the
55 needle is subjected to a constant laminar air flow which acts as an air knife. In use, with the syringe full of solution containing the material to be encapsulated, the stepping pump is actuated to incrementally force droplets of solution from the
60 tip of the needle. Each drop is "cut off" by the air stream and falls approximately 2.5 cm into the $CaCl_2$ solution where it is immediately gelled by absorption of calcium ions. The distance between the tip of the needle and the surface of the $CaCl_2$
65 solution is great enough, in this instance, to allow

the sodium alginate/cell suspension to assume the most physically favorable shape; a sphere (maximum volume for minimum surface area). Air within the tube bleeds through an opening in the
70 stopper. This results in "cross-linking" of the gel and in the formation of a high viscosity shape-retaining protective temporary capsule containing the suspended tissue and its medium. The capsules collect in the solution as a separate
75 phase and may be separated by aspiration.
    In the next step of the process, a semipermeable membrane is deposited about the surface of the temporary capsules by "cross-linking" surface layers. This may be effected by
80 subjecting the gelled temporary capsules to an aqueous solution of a polymer containing cationic groups reactive with anionic functionalities in the gel molecules. Polymers containing acid reactive groups such as free imine or amine groups are
85 preferred. In this situation, the polysaccharide gum is crosslinked by interaction (salt bond formation) between the carboxyl groups and the amine or imine groups. Permeability can be controlled within limits by selecting the molecular weight of
90 the cross-linking polymer used and by regulating the concentration of the polymer solution and the duration of exposure. A solution of polymer having a low molecular weight, in a given time period, will penetrate further into the temporary capsules than
95 will a high molecular weight polymer. The degree of penetration of the cross-linker has been correlated with the resulting permeability. In general, the higher the molecular weight and the less penetration, the larger the pore size. Broadly,
100 polymers within the molecular weight range of 3,000 to 100,000 daltons or greater may be used, depending on the duration of the reaction, the concentration of the polymer solution, and the degree of permeability desired. One successful set
105 of reaction conditions, using polylysine of average molecular weight of about 35,000 daltons, involved reaction for two minutes, with stirring, of a physiological saline solution containing 0.0167 percent polylysine. This results in membranes
110 having an upper limit of permeability of about 100,000 daltons. Optimal reaction conditions suitable for controlling permeability in a given system can readily be determined empirically in view of the foregoing guidelines. Using this
115 method it is possible to set the upper permeability limit of the membranes at a selected level below about 200,000 daltons.
    Examples of suitable cross-linking polymers include proteins and polypeptides, either natural
120 or synthetic, having free amino or imino groups, polyethyleneamines, polyethyleneimines, and polyvinyl amines. Polylysine, in both the D and L forms, has been used with success. Proteins such as polyargenine, polycitrulline, or polyornithine are
125 also operable. Polymers in the higher range of positive charge density, e.g., polyvinylamine, vigorously adhere to the anionic groups of the gel molecules to form stable membranes, but the membranes are rather difficult to disrupt.
130   Treatment with a dilute solution of gum will tie

up free amino groups on the surfaces of the capsules which otherwise may impart to the capsules a tendency to clump.

5   At this point in the encapsulation, capsules may be collected which comprise a semipermeable membrane surrounding a gelled solution of gum, cell-type compatible culture medium, cells, and optionally an internal matrix of collagen or another anchorage substrate. Since mass transfer should
10   be promoted within the capsules and across the membranes, it is preferred to reliquefy the gel to its water-soluble form. This may be done by re-establishing the conditions under which the gum is a liquid, e.g., removing the calcium or other
15   multifunctional cations from the interior gel. The medium in the capsule can be resolubilized simply by immersing the capsules in phosphate buffered saline, which contains alkali metal ions and hydrogen ions. Monovalent ions exchange with
20   the calcium or other multifunctional ions within the gum when the capsules are immersed in the solution with stirring. Sodium citrate solutions may be used for the same purpose, and serve to sequester the divalent ions.

25   Cell cultures encapsulated as described above may be suspended in culture media designed specifically to satisfy all of the requirements of the particular cell type involved and will continue to undergo normal *in vitro* metabolism. If the culture
30   requires an environment of high molecular weight components such as serum components, these may be omitted from the extracapsular medium. Typically, the components normally ingested by cells are of relatively low molecular weight and
35   readily diffuse across the capsule membranes into the microenvironment of the cells where they permeate the cell membrane. Products of metabolism of the cells which are secreted into the intracapsular medium, if they have a molecular
40   weight below the upper limit of permeability of the capsule membrane, likewise diffuse thereacross and collect in the extracapsular medium.

The encapsulated cells may be cultured under conditions of, e.g., temperature, pH, and ionic
45   environment, identical to conventional cultures. Also, cell-produced products may be harvested from the extracapsular medium or from the capsules by conventional techniques. However, the culturing technique disclosed herein has the
50   following advantages:

1. The cells of the culture are protected from shearing forces and mechanical damage and from contamination by factors having dimensions in excess of the upper permeability limit of the
55   membranes. This means that handling and sterility requirements normally incident to culturing procedures can be somewhat relaxed, since microorganisms cannot reach the encapsulated cells, and virus infected cells need not
60   contaminate other cells.

2. The capsules in effect immobilize the cells within an environment in which enclosed high molecular weight materials are confined, yet lower molecular weight cell nutrients and products are readily introduced and removed. This allows the

nutrient both to be intermittently or continuously collected and supplemented as desired, without disturbing the cells.

3. Substances of interest produced by the cells
70   are more easily recovered. Secreted cell products of molecular dimensions small enough to permeate the capsule membranes collect in the extracapsular medium in admixture with nutrients. However, high molecular weight serum
75   components and the like are not released into the extracapsular medium, thus simplifying recovery of a cell product of interest. Secreted cell products of molecular dimensions in excess of the upper permeability limit of the membranes collect within
80   the capsules. Of course, cell products not secreted through the cell membrane may also be of interest. These may be recovered in relatively concentrated form by isolating the capsules and subsequently selectively disrupting the capsule
85   membranes using, for example, the technique disclosed hereinafter, and if necessary by disrupting the cell membranes.

4. The intracapsular volume provides an environment well suited for cell division.
90   Suspension cultures have been observed to undergo mitosis within the capsule. Anchorage dependent cells which in normal cultures grow in a two-dimensional monolayer multiply to form an array within the capsule. Such cells use the
95   interior surfaces of the membrane as a substrate and/or anchor to the high molecular weight materials set forth above which are disposed within the capsule. This leads to significant increases in cell density as compared with
100   conventional cultures. The ongoing viability of such cell clusters is aided by the fact that the surface area to volume ratios of the capsules can be quite large, and thus all cells have access to required nutrients, oxygen, etc.
105   In certain situations it would be advantageous to selectively disrupt the capsule membranes to release the cells without damage. One notable example is in the production of interferon (INF). Cells capable of producing INF must be subjected
110   to certain viruses or nucleic acids in preparation for the INF production stage. Also, in several INF induction procedures, reagents are added to the culture to inhibit protein synthesis. Accordingly, the growth stage of the culturing process must be
115   conducted under conditions quite different from the INF induction stage. If the substances used for INF induction are of a molecular weight in excess of the upper permeability limit of the capsule membranes (as will be the case in virus
120   inductions) the induction process cannot be accomplished in the encapsulated cell culture. Accordingly, INF producing cells, if grown within the capsule, would have to be released by disruption of the membrane in order to be
125   subjected to the induction process.

Disruption of Membranes

Cells confined in membranes of the type set forth above may be released by a process involving commercially available reagents having

properties which do not significantly adversely affect the encapsulated cells. First, the capsules are separated from their suspending medium, washed thoroughly to remove any contaminants present on the exterior of the microcapsules, and then dispersed, with agitation, in a mixed solution of monatomic, multivalent cations such as calcium ions and a stripping polymer having plural anionic moieties such as a salt of a polysulfonic or polyphosphoric acid. Heparin, a natural sulfonated polysaccharide, is preferred for this step. The anionic charge density of the stripping polymer used should be equal to or preferably greater than the charge density of the polyanionic material originally employed to form the membranes. The molecular weight of the polymer should be at least comparable to and preferably greater than the molecular weight of the polymer having plural cationic groups used in forming the membrane. Within the suspension of capsules in the mixed solution, the calcium ions compete with the polycationic polymer chains used to form the membrane for anionic sites on the water-soluble gum. Simultaneously, the heparin or other polymer having plural anionic moieties dissolved in the solution competes with the gum in the membrane for cationic sites on the polymer chains. This results in a water-dispersable or preferably water-soluble complex of e.g., polylysine and heparin, and in association of the monatomic cations with molecules of the gel.

This step renders the membrane susceptible to dissolution upon subsequent exposure to a sequestering agent which completes the disruption process by taking up monatomic ions from the gel. Capsule membrane debris which remains in the medium, if any, can be easily separated from the cells.

The currently preferred solution for the first stage of the selective disruption process comprises 1.1% calcium chloride (w/v) and between 500 to 1,500 units of heparin per milliliter of solution. A volume of microcapsules is added to this solution sufficient to constitute between about 20% and 30% of the total volume of suspension. Calcium chloride and heparin are preferred for disrupting membranes of cell-containing capsules since both reagents are physiologically compatible with most cells and therefore minimize the possibility of cell damage. Mixtures of aluminum salts or other multivalent cations (but not Mg^{++} ions) may also be used together with the polysulfonic or polyphosphoric acid salts of the type set forth above.

In general, the concentrations of monatomic ions and anionic polymer used in this step may vary widely. Optimum concentrations may be readily determined empirically, and depend on exposure time as well as the particular polymer used to form the membranes.

The currently preferred sequestering agent for performing the selective disruption is sodium citrate, although other alkali metal citrate salts and alkali metal EDTA salts may also be used. When sodium citrate is employed, the optimum

concentration is on the order of 55 mM. It is preferred to dissolve the citrate or other sequestering agent in isotonic saline so as to minimize cell damage.

The invention will be further understood from the following non-limiting examples.

EXAMPLE 1: INSULIN PRODUCTION

Islets of Langerhans are obtained from human cadaver of animal pancreas and added to a complete tissue culture (CMRL-1969 Connaught Laboratories, Toronto, Canada) at a concentration of approximately 10^3 islets per milliliter. The tissue culture contains all nutrients needed for continued viability of the islets as well as the amino acids employed by the Beta cells for making insulin. Four-tenths of a milliliter of a 10^3 islet per milliliter suspension is then added to a one-half milliliter volume of 1.2 percent sodium alginate (Sigma Chemical Company) in physiological saline.

Next, a 1.5 percent calcium chloride solution is used to gel droplets of the solution formed as set forth above. Droplets on the order of 300—400 microns in diameter emanating from the tip of the needle immediately gel upon entering the calcium solution. The gelled capsules are then transferred to a beaker containing 15 ml of a solution comprising one part of a 2% 2 (cyclohexylamino) ethane sulfonic acid buffer solution in 0.6% NaCl (isotonic, ph = 8.2) diluted with 20 parts 1% $CaCl_2$. After a 3 minute immersion, the capsules are washed twice in 1% $CaCl_2$.

The capsules are then transferred to a 32 ml solution comprising 1/80 of one percent polylysine (average MW 35,000 daltons) in physiological saline. After 3 minutes, the polylysine solution is decanted. The capsules are washed with 1% $CaCl_2$, and optionally resuspended for 3 minutes in a solution of polyethyleneimine (MW 40,000—60,000) produced by diluting a stock 3.3% polyethyleneimine solution in morpholino propane sulfonic acid buffer (0.2M, ph = 6) with sufficient 1% $CaCl_2$ to result in a final polymer concentration of 0.12%. The resulting capsules, having "permanent" semipermeable membranes, are then washed twice with 1% $CaCl_2$, twice with physiological saline, and mixed with 10 ml of 0.12 percent alginic acid solution.

The capsules resist clumping, and many can be seen to contain islets of Langerhans. Gel on the interior of the capsules is reliquefied by immersing the capsules in a mixture of saline and citrate buffer (pH —7.4) for 5 minutes. Lastly, the capsules are suspended in CMLR-69 medium.

Under the microscope, these capsules are seen to consist of a very thin membrane which encircles an islet within which individual cells can be seen. Molecules having a molecular weight up to about one-hundred thousand can traverse the membranes. This allows oxygen, amino acids, nutrients, and plasma components used in culture media (i.e., lower molecular weight fetal calf plasma components) to reach the islet and allows insulin to be secreted.

After repeated washings in physiological saline, microcapsules made in accordance with the above procedure containing approximately 15 islets are suspended in 3 milliliters of CMRL-1969. When 5 eight days old, in the presence of 600 mg/dl glucose, the capsules secreted into the extracapsular medium, in one run, 67 units/ml insulin in 1.5 hours. In a second run, 68 units/ml insulin were produced in the same amount of 10 time. One week old capsules, in the same medium, but in the presence of 100 mg/dl glucose, in a first run, secreted 25 units/ml insulin in 1.2 hours, and in a second run, secreted 10 units/ml.

EXAMPLE 2: INF-β PRODUCTION
15   Human fibroblasts obtained by treating human foreskin tissue with trypsin and EDTA for 5 minutes at 37°C in a known manner are suspended in a complete growth medium (CMLR-1969, Connaught Laboratories) supplemented 20 with 40% (v/v) purified fetal calf serum, 0.8% sodium alginate (Sigma) and 200 µg/ml purified calf skin collagen. The density of the cell suspension is about 1.5×10^7 cells/ml. Temporary alginate capsules are formed as set 25 forth in Example 1. Semipermeable membranes are deposited in surface layers of the capsules by suspending them in a 0.005% (w/v) aqueous solution of polylysine (MW 43,000 daltons) for 3 minutes.
30   The resulting capsules are suspended in CMLR-1969 supplemented with 10% fetal calf serum. The foregoing steps are all conducted at 37°C. After incubation at the same temperature, the capsules, if examined under the microscope, will 35 be found to contain fibroblasts which have undergone mitosis and display three-dimensional fibroblastic morphology within the microcapsules.
    After 4—5 days of incubation, the encapsulated fibroblasts are subjected to an INF-β 40 superinduction technique according to the Vilcek procedure. Under a 5% CO_2 atmosphere (95% air), the capsule suspension is incubated at 37°C for one hour in the presence of 100 µg/ml Poly I-Poly C, a double stranded RNA (known INF-β inducer) 45 available from PL Biochemicals, Milwaukee, Wisconsin and 50 µg/ml cycloheximide (protein synthesis inhibitor, Calbiochem, La Jolla, California). After one hour, the suspended capsules are washed in medium (CMLR-1969) 50 containing 50 µg/ml cycloheximide and then resuspended in the same solution for 3 hours at 37°C under a 5% CO_2 atmosphere. At the completion of this incubation the washing step is repeated and the capsules are resuspended in 55 medium containing 50 µg/ml cycloheximide and 5 µg/ml actimomycin D (a known RNA synthesis inhibitor, Calbiochem) and incubated for 2 hours at 37°C under a 5% CO_2 atmosphere. The capsules are then washed twice in medium and 60 suspended in serum-free medium at 37°C for 18—24 hours, during which time the fibroblasts secrete INF-β, which has a molecular weight on the order of 21,000 daltons and may be harvested from the extracapsular medium.

65 EXAMPLE 3: INF-β PRODUCTION
    The procedure of Example 2 is repeated, except that prior to induction the capsule membranes are selectively disrupted so that the Poly I·Poly C can more easily gain access to the fibroblasts. The 70 disruption procedure is conducted as follows.
    10 ml portions of microcapsule suspensions containing about 500—1000 capsules per ml are allowed to settle and the suspension medium is aspirated off. The capsules are washed twice with 75 phosphate buffered saline (PBS, pH = 7.4). The washed capsules are then mixed with a 3.0 ml aliquot of PBS containing 1000 units/ml heparin and 1.1% (w/v) $CaCl_2$. The suspension is agitated at 37°C for 3 minutes, after which the capsules 80 are allowed to settle, the supernatant is aspirated off, and the capsules are washed twice with 3.0 ml of 0.15M NaCl. After aspiration of the second wash solution, the capsules are mixed with 2.0 ml of a mixed solution comprising equal 85 volumes of 110 mM sodium citrate and 0.15M NaCl (pH = 7.4). The mixture is hand vortexed for 1 minute to induce dissolution of the membranes after which cells are washed twice in medium.
    The cells are then suspended in medium, 90 subjected to the induction procedure set forth in example 2, and then re-encapsulated as set forth in Example 2. The capsule suspension is then incubated in serum-free medium for 18—24 hours, during which time INF-β is secreted from 95 the cells, permeates the capsule membranes, and collects in the extracapsular medium.
    Examples 2 and 3, if conducted with Poly I-Poly C (5S) (sedimentation value, Poly I and Poly C annealed to form double stranded RNA) result in 100 the following INF-β production levels, in units of INF-β/10^5 cells:

	1.	2.
Example 2	$\frac{-}{25}$	$\frac{-}{25}$
Example 3	2,500	2,500

105   Examples 2 and 3, if conducted with Poly I-Poly C (12S) (sedimentation value, double stranded as purchased) result in the following INF-β production levels, in units of INF-β/10^5 cells:

	1.	2.
Example 2	$\frac{-}{25}$	$\frac{-}{25}$
Example 3	2,500	2,500

    The one-hundred fold increase in production using the procedure of Example 3 over that of Example 2 is believed to be due, at least in part, to the fact 115 that the Poly I·Poly C has better access to the cells in the Example 3 procedure.

EXAMPLE 4
    The procedure of Example 2 is repeated except that capsules containing no collagen are 120 employed. The encapsulated cells were grown in

conventional monolayer culture, treated with trypin, and induced with Poly I·Poly C (5S) and microencapsulated simultaneously. The extracapsular medium is found to contain 2,500 5 units INF-β/10^5 cells.

EXAMPLE 5: MONOCLONAL ANTIBODIES
    Hybridoma cells obtained from Herman Eisen at MIT were cultured to a density of 3.0 × 10^6 cells/ml. These cells had been fused 10 from mouse spleen cells and mouse myeloma cells in a manner now well known in the prior art and constituted an immortal cell line which in culture produced antibodies agains dinitrophenyl bovine serum albumin. Three ml aliquots of the 15 cell suspension were made up by adding 2.1 ml of suspension containing 1.4% sodium alginate to 0.6 ml fetal calf serum and 0.3 ml physiological (150 mM) saline. Droplets of the suspension were immediately gelled in CaCl$_2$ solution and then 20 treated with a 0.016 weight percent solution of poly L lysine. The interior of the resulting capsules was then reliquefied by immersion in a solution of one part 110 mM sodium citrate and three parts 150 mM saline for 6 minutes. The capsules 25 containing hybridoma cells were then suspended in a mixture of RPMI-1640 medium (Gibco) containing 20% heat inactivated fetal calf serum.
    Cell counts of encapsulated and unencapsulated hybridoma cultures, and the 30 amount of monoclonal antibody produced by both the encapsulated and unencapsulated cultures were determined periodically. The results are set forth in graphical form in Fig. 2.

EXAMPLE 6: INF-α FROM LEUKOCYTES
35   30 ml buffy coats obtained from the American Red Cross were treated with 3.0 ml of 5% EDTA and repeated 10 minute exposures to 0.83% NH$_4$Cl at 4°C to lyse the red cells. A five minute centrifuge (1200 rpm at 4°C) between NH$_4$Cl 40 treatments separated debris from the remaining intact leukocytes. The cells were next suspended in MEM (minimum essential medium, serum free — Gibco), diluted by a factor of 100, and stained with tryptan blue for 15 minutes. A cell count 45 conducted on a sample showed that about 1.3 × 10^8 leukocytes per 30 ml buffy coat survived. The cells were then suspended at a density of 1 × 10^7 cells/ml in medium supplemented with 2% heat inactivated fetal calf 50 serum.
    Induction was effected by exposing the cell suspension to Sendai virus (various concentrations in haemagglutinating units/ml — Flow Laboratories, Md.) for one hour at 37°C with 55 stirring. The virus was then separated from the cell by centrifugation at room temperature and the cells were resuspended in equal volumes of MEM — 4% heat inavtivated fetal calf serum and 1.4% sodium alginate. Capsules were formed as set 60 forth above and then resuspended in serum-free and serum-containing media. There were no significant differences in the quantities of INF detected in the extracapsular medium of these

test samples. INF production levels were also 65 identical in unencapsulated control cultures. The results of these experiments are set forth below

	Units Sendai Virus (HA Units/ml)	INF Produced Units 10^7 Cells
	600	10
70	300	20
	150	33
	75	50

EXAMPLE 7: INF-α FROM LYMPHOBLASTOIDS
    Namalwa cells from the American Type Culture 75 Collection were grown both in conventional culture and within microcapsules in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum. Volumes of the cell suspensions were then subjected to INF induction and 80 production procedures, with one volume encapsulated and the other unencapsulated. The cultures contained substantially equal numbers of cells. To both the encapsulated and unencapsulated cultures was added 25 mg/ml 85 bromo deoxyuridine in double distilled water to inhibit mitosis. After incubation for 36 hours at 37°C, the cells of both cultures were washed and then suspended in RPMI-1640 medium supplemented with 2% heat inactivated fetal calf 90 serum.
    The encapsulated culture was then treated to selectively disrupt the capsule membranes. The capsules were washed three times in physiological saline incubated in 1000 units/ml heparin solution 95 containing 1.1% CaCl$_2$ for 10 minutes at 37°C, and then rewashed in saline. The washed capsules are next incubated for 5 minutes at 37°C with dilute sodium citrate solution in physiological saline. Agitation of the capsule suspension at this 100 point results in dissolution of the membranes and release of the Namalwa cells. The cel suspension is then centrifuged to remove debris and washed several times in citrate/saline solution.
    Both cultures were next suspended in fresh 105 RPMI-1640 culture medium supplemented with 2% heat inactivated fetal calf serum and buffer (pH × 7.4) at a density of 1.0 × 10^6 cells/ml.
    To both the conventional culture and the formerly encapsulated culture were then added 110 the Bankowski strain of Newcastle Disease Virus in amniotic fluid. The virus was at a concentration of 1.0 × 10^8 pfu/ml and was purchased from Poultry Health Laboratories, Davis, California. One ml of the virus was added for each 10 ml of cell 115 suspension. The cultures were incubated for 24 hours at 37°C.
    The conventional culture was then divided into five parts (1—5 below); the formerly encapsulated culture was divided into 4 parts (6—9 below). 120 Each of the 9 aliquots of culture were then assayed for INF production following the treatments set forth below.
        1. untreated
        2. resuspended in RPMI-1640 medium with 2% 125 heat inactivated fetal calf serum

3. resuspended in RPMI-1640 medium serum-free

4. encapsulated together with RPMI-1640 medium and 5% heat-inactivated fetal calf serum-capsules suspended in serum-free medium

5. encapsulated together with RPMI-1640 medium and 5% heat-inactivated fetal calf serum-capsules suspended in medium with 2% fetal calf serum

6. resuspended in serum-free medium

7. resuspended in medium containing 2% heat-inactivated fetal calf serum

8. reencapsulated together with medium plus 5% heat-inactivated fetal calf serum-capsules suspended in serum-free medium

9. reencapsulated together with medium plus 5% heat-inactivated fetal calf serum-capsules suspended in medium plus 2% serum

The following table sets forth the quantity of cells required in each of the cell cultures 1—9 to produce 1 unit of INF-α:

1	30	6	40
2	45	7	40
3	—	8	1000, 360
4	680	9	200, 100
5	2000		

CLAIMS

1. A process for producing a substance which is produced by living cells, wherein the process comprises the steps of:

A. encapsulating the cells within membranes having a selected upper limit of permeability;

B. suspending the encapsulated cells in an aqueous culture medium;

C. allowing said cells to undergo metabolism in vitro and to secrete the required substance; and

D. harvesting the substance either from the aqueous culture medium or from within the membranes.

2. The process according to claim 1, wherein encapsulation step (A) is effected by forming the membrane by reaction between cationic groups on polymer chains and anionic groups on a water-soluble gum to form a water-insoluble salt bonded matrix.

3. The process according to claim 1, wherein encapsulation step (A) is effected by:

1) suspending the cells in an aqueous medium physiologically compatible therewith and containing a water-soluble gum having plural anionic moieties;

2) forming the suspension into droplets containing the cells;

3) subjecting the droplets to a solution containing multivalent, physiologically compatible cations to gel the droplets as discrete, shape-retaining, water-insoluble temporary capsules; and

4) cross-linking surface layers of said temporary capsules to produce semipermeable membranes about said droplets by subjecting them to a polymer comprising plural cationic groups reactive with said anionic moieties.

4. The process according to claim 3, comprising the additional step of resolubilizing the gel within the membrane produced in step 4).

5. The process according to claim 1, 2, 3 or 4, wherein the required substance has a molecular weight such that the substance is capable of traversing the membrane, and the process comprises allowing the substance to diffuse through the encapsulating membranes into the aqueous medium and harvesting the substance therefrom.

6. The process according to any of claims 1 to 5, wherein the cells are encapsulated together with a complete cell culture medium sufficient to maintain the cells and to allow biosynthesis of the substance in vitro.

7. The process according to any of claims 1 to 5, wherein the aqueous medium used in step (B) is a complete cell culture medium sufficient to maintain the cells and to allow biosynthesis of the substance in vitro.

8. The process according to claim 6, wherein a component having a molecular weight such that the component is unable to traverse the said membranes is needed by the cells in order to allow in vitro biosynthesis of the said substance, and the process comprises the additional step of encapsulating the said component together with the cells.

9. The process according to claim 8, wherein the aqueous culture medium of Step B is substantially free of the said component.

10. The process according to any of claims 1 to 9, wherein the cells are mammalian cells.

11. The process according to any of claims 1 to 9 or 10, comprising the additional step of allowing the cells to undergo mitosis within their capsules.

12. The process according to any of claims 1 to 9, wherein the cells are cells which have been genetically modified.

13. The process according to any of claims 1 to 12, wherein encapsulation step (A) is conducted under conditions producing spheroidal microcapsules having a diameter below about 0.5 mm.

14. The process according to any of claims 1 to 13, wherein the substance harvested in step (E) is selected from insulin, glucagon, prolactin, somatostatin, thyroxin, hormones including steroid hormones and pituitary hormones, follicle-stimulating hormone and PTH, interferons, lymphokines and antibodies.

15. The process according to any preceding claim, wherein the cells comprise hybridoma cells, and the said substance comprise monoclonal antibodies having a molecular weight such that the antibodies cannot traverse the membranes, the antibodies being harvested from within said membranes.

16. The process according to any preceding claim, wherein said selected upper limit of permeability is below about 1.5×10^5 daltons.

17. A process employing encapsulated living cells to produce desired substances biologically, substantially as herein described by way of

example.

18. A system for culturing living cells comprising encapsulated viable cells suspended in an aqueous culture medium, membranes
5 encapsulating the cells being characterised by an upper limit of permeability sufficient to allow traverse of nutrients required by the cells, the membranes enclosing suspended viable cells disposed in a medium which includes all
10 components needed to maintain viability of said cells which are of a size beyond the upper permeability limit of the membranes, and the aqueous culture medium comprising all components needed to maintain viability of said
15 cells which have a molecular size below the upper permeability limit.

19. The system according to claim 18, wherein the first-mentioned components comprise serum components.

20 20. The system according to claim 18, wherein the cells comprise mammalian cells.

21. The system according to claim 18, wherein the cells comprise microorganisms.

22. The system according to claim 18, wherein
25 the cells comprise genetically modified cells.

23. The system according to claim 18, wherein the cells comprise hybridoma cells.

24. The system according to claim 18, wherein the cells comprise cells capable of secreting in
30 vitro a substance selected from hormones, interferons, lymphokines, and antibodies.

25. The system according to claim 18, wherein the cells comprise cells capable of secreting in vitro a substance selected from insulin, glycogen,
35 growth hormones, pituitary hormones, steroid hormones, prolactin, somatostatin, PTH, and FSH.

26. The system according to any of claims 18 to 25, wherein the porous membranes comprise a polymer gum having plural anionic moieties salt
40 bonded to a polymer having plural cationic moieties.

27. A system according to claim 18 and substantially as herein described with reference to Fig. 1 of the accompanying drawings.

45 28. A system according to claim 18 and as described in any one of Examples 1 to 7.

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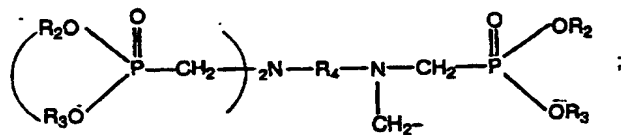**Nerima-ku, Tokyo (JP)**

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(54) Polyphenylene ether resin compositions and shaped articles thereof.

(57) A polyphenylene ether resin composition comprises a polyphenylene ether resin matrix and dispersed therein as heat stabilizer a phosphonic acid or derivative thereof of the formula

(I)

wherein R_1 represents an unsubstituted or substituted phenyl or naphthyl group, or a linear or branched alkyl group of 1 to 18 carbon atoms, or a group of the formula (a)

R_2 and R_3 are the same or different and each represents hydrogen atom, a metal, an unsubstituted or substituted phenyl group, or an alkyl group of 1 to 10 carbon atoms and R_4 is a divalent hydrocarbon group of 2 to 10 carbon atoms.

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DESCRIPTION

"POLYPHENYLENE ETHER RESIN COMPOSITIONS AND
SHAPED ARTICLES THEREOF"

This invention relates to a polyphenylene
ether resin composition having improved heat stability.

Polyphenylene ethers are known resins which are
disclosed, for example, in U. S. Patents Nos. 3306874,
5   3306875, 3257357, 3257358 and 4011200 and Japanese Laid-
Open Patent Publication No. 126800/1975. Since poly-
phenylene ethers having a molecular weight above a
certain limit have a high softening point, they are
useful in applications which require heat stability. In
10 formulating a polyphenylene ether into resin compositions,
however, its high softening point makes it necessary to
use higher kneading and extruding temperatures than in
the case of other versatile resins, and high temperatures
are also required in molding the resin compositions.
15 Moreover, molded articles of the polyphenylene ether resin
compositions are frequently used at relatively high
temperatures over long periods of time in contrast to
those from versatile resins.

Because polyphenylene ethers are relatively
20 unstable to heat as is well known, they undergo degradation
during extrusion and molding at high temperatures, and
result in polyphenylene ether resin compositions and
molded articles having degraded properties and/or dis-
coloration. These deleterious effects limit widespread
25 utilization of polyphenylene ether resin compositions,
and it has been desired to remedy these defects, particu-
larly to improve their heat stability at high temperature.

Various methods have already been proposed for

the stabilization of resin compositions containing poly-
phenylene ethers. These methods are classified into a
group involving capping the hydroxyl groups present at
the terminals of the polyphenylene ether molecule by
5 acylation, etc., and a group comprising adding various
stabilizers to polyphenylene ethers.

Known stabilizers used in the latter group
include, for example, benzoates (U. S. Patent No.
3,379,875), hexa-alkylphosphoric triamides or combinations
10 thereof with other compounds (U. S. Patents Nos. 3414536,
3420792, 3429850, 3465062, 3472814, 3483271, 3792121 and
3816562), octa-alkylpyrophosphoramides or combinations
thereof with other compounds (U. S. Patent No. 3,450,670),
amines (U. S. Patents Nos. 3,563,934 and 3,956,423),
15 phosphites or hydrazines (U. S. Patent No. 3,639,334).
alkanolamines (U. S. Patent No. 3,761,541), arylphosphonic
amides (U. S. Patent No. 3,792,120), sterically hindered
phenols having a triazine or isocyanuric ring (U. S. Patent
No. 4,154,719), substituted dicarboxylic acid dihydrazides
20 (U. S. Patent No. 3,954,904), high-molecular-weight
phosphites or combinations thereof with other compounds
(U. S. Patent No. 3,952,072), amides (Japanese Patent
Publication No. 29748/1969), metal dithiocarbamates
(Japanese Patent Publications Nos. 19395/1970 and 8352/
25 1970), carboxylic acid anhydrides (Japanese Patent
Publication No. 29,750/1969), phosphites (Japanese Patent
Publication No. 29,751/1969), sterically hindered phenols
or combinations thereof with other compounds (Japanese
Patent Publicatoons Nos. 43473/1971, 42029/1971, 42030/
30 1971, 42031/1971, 42032/1971, and 42033/1971), sterically
hindered phenols having one amide linkage in the molecule
(Japanese Patent Publication No. 24782/1971), sterically
hindered phenols having one ester linkage in the molecule
(Japanese Patent Publication No. 38623/1973), high-
35 molecular-weight phosphites (Japanese Laid-Open Patent
Publications Nos. 23846/1974, 31755/1974 and 40476/1975),
and combinations of phosphorous acid amides and boron
compounds (Japanese Laid-Open Patent Publication No.
129750/1974).

None of these numerous stabilizers previously proposed have been conductive to the provision of poly-phenylene ether resin compositions having fully satis-factory heat stability, particularly at high temperatures,

5   in practical applications.

It is an object of this invention therefore to improve the heat stability of a polyphenylene ether resin composition.

Another object of this invention is to provide

10 a polyphenylene ether resin composition having excellent heat stability at high temperatures.

Still another object of this invention is to provide a polyphenylene ether resin composition showing inhibited degradation against a long heat history at high

15 temperatures, which can withstand high temperatures during kneading, extrusion and molding and give molded articles having excellent heat stability in long-term use at high temperatures.

Other objects and advantages of this invention

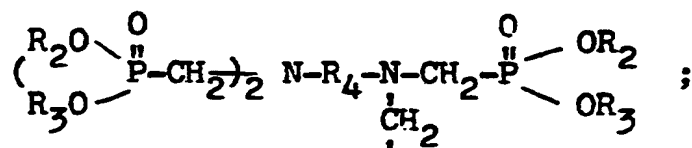20 will become apparent from the following description.

In accordance with this invention, the objects and advantages of this invention are achieved by a poly-phenylene ether resin composition having improved heat stability, said composition comprising a polyphenylene

25 ether resin matrix and dispersed therein, a phosphonic acid or its derivative represented by the following formula (I)
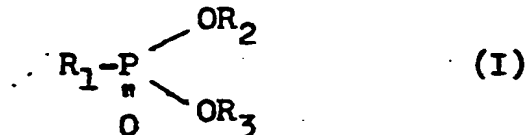
$$R_1-P \begin{matrix} \diagup OR_2 \\ \diagdown OR_3 \end{matrix} \quad (I)$$
$$\underset{O}{\overset{\text{\tiny}}{}}$$

wherein R_1 represents an unsubstituted or

30 substituted phenyl or naphthyl group, or a linear or branched alkyl group with 1 to 18 carbon atoms, or a group of the following formula (a)

$$\left(\begin{matrix} R_2O \\ R_3O \end{matrix} \diagdown \overset{\overset{O}{\parallel}}{P}-CH_2 \right)_2 N-R_4-N-CH_2-\overset{\overset{O}{\parallel}}{P} \diagup\diagdown \begin{matrix} OR_2 \\ OR_3 \end{matrix} \quad ; $$
$$\underset{\underset{CH_2}{\mid}}{\mid}$$

R_2 and R_3 are identical or different and
each represents hydrogen atom, a metal, an
unsubstituted or substituted phenyl group,

5   or an alkyl group with 1 to 10 carbon atoms;
and R_4 is a divalent hydrocarbon group with
2 to 10 carbon atoms.

According to this invention, there is preferably
provided a polyphenylene ether resin composition having

10   improved heat stability, comprising a polyphenylene ether
resin matrix and dispersed therein, both the aforesaid
phosphonic acid or its derivative and a sterically
hindered phenol.

According to an especially preferred aspect of

15   this invention, there is provided a polyphenylene ether
resin composition having improved heat stability, compris-
ing a polyphenylene ether resin matrix and dispersed
therein, an organic monophosphite or an organic poly-
phosphite as well as the aforesaid phosphonic acid or

20   its derivative and the aforesaid sterically hindered
phenol.

The polyphenylene ether resin forming the resin
matrix in the composition of this invention can be a
polyphenylene ether homopolymer or copolymer obtained by

25   polycondensing at least one mononuclear phenol of the
formula

$$R_7 \diagdown \overset{OH}{\underset{R_6}{\bigcirc}} \diagup R_5 \qquad \text{(II)}$$
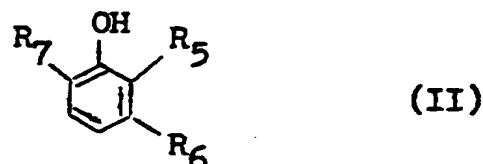
wherein R_5, R_6 and R_7, independently from
each other, represent a hydrogen atom or

30   an alkyl group having 1 to 3 carbon atoms,

provided that at least one of R_5 and R_7
is an alkyl group having 1 to 3 carbon
atoms,

or a grafted polyphenylene ether obtained by graft-

5   polymerizing such a polyphenylene ether with a vinyl
aromatic compound.

Methods for producing these polyphenylene
ethers are well known per se.

Examples of the mononuclear phenols of general
10   formula (II) include 2,6-dimethylphenol, 2,6-diethylphenol,
2,6-dipropylphenol, 2-methyl-6-ethylphenol, 2-methyl-6-
propylphenol, 2-ethyl-6-propylphenol, m-cresol, 2,3-
dimethylphenol, 2,3-diethylphenol, 2,3-dipropylphenol,
2-methyl-3-ethylphenol, 2-methyl-3-propylphenol, 2-ethyl-
15   3-methylphenol, 2-ethyl-3-propylphenol, 2-propyl-3-
methylphenol, 2-propyl-3-ethylphenol, 2,3,6-trimethyl-
phenol, 2,3,6-triethylphenol, 2,3,6-tripropylphenol,
2,6-dimethyl-3-ethylphenol, and 2,6-dimethyl-3-propyl-
phenol.

20   Polyphenylene ethers derived from these mono-
nuclear phenols, therefore, include homopolymers such as
poly(2,6-dimethyl-1,4-phenylene)ether, poly(2,6-diethyl-
1,4-phenylene)ether, poly(2,6-dipropyl-1,4-phenylene)ether,
poly(2-methyl-6-ethyl-1,4-phenylene)ether, poly(2-methyl-
25   6-propyl-1,4-phenylene)ether, and poly(2-ethyl-6-propyl-
1,4-phenylene)ether; and copolymers such as a 2,6-
dimethylphenol/2,3,6-trimethylphenol copolymer (which
denotes a polyphenylene ether copolymer derived from
2,6-dimethylphenol and 2,3,6-trimethylphenol, and in the
30   following description, polyphenylene ether copolymers are
represented in the same manner),a 2,6-dimethylphenol/
2,3,6-triethylphenol copolymer, a 2,6-diethylphenol/
2,3,6-trimethylphenol copolymer and a 2,6-dipropylphenol/
2,3,6-trimethylphenol copolymer.

35   The grafted polyphenylene ethers used equally
to these homopolymers and copolymers in this invention
are obtained by grafting vinyl aromatic compounds such as

styrene, alpha-methylstyrene, vinyltoluene and vinylxylene
to these homopolymers or copolymers, and include, for
example, styrene-grafted poly(2,6-dimethyl-1,4-phenylene)-
ether, and a styrene-grafted 2,6-dimethylphenol/2,3,6-

5   trimethylphenol copolymer.

Preferably, such grafted polymers have a grafting
ratio of about 10 to about 50%, especially about 20% to
about 40%.

Among these polyphenylene ethers, poly(2,6-

10   dimethyl-1,4-phenylene)ether, a 2,6-dimethylphenol/2,3,6-
trimethylphenol copolymer, and grafted polyphenylene
ethers obtained by grafting styrene to such polymers are
especially preferred for use in this invention.

The resin matrix in the composition of this

15   invention may be composed of such a polyphenylene ether
alone, or a mixture of it with another polymer. The
other polymer may be a thermoplastic resin or an elastomer.

The thermoplastic resin as referred to herein
is a resin containing at least 25% by weight of a recurring

20   structural unit of the following general formula (III)
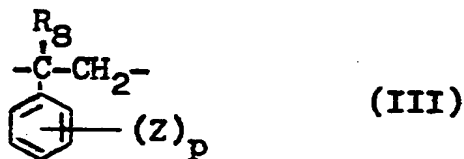
$$-\overset{\displaystyle R_8}{\underset{}{\underset{\displaystyle \bigotimes\!\!-(Z)_p}{C}}}-CH_2- \qquad (III)$$

wherein R_8 represents a hydrogen atom or a
lower alkyl group, Z represents a halogen
atom or a lower alkyl group, and p is 0 or

25   a positive integer of 1 to 3,
in the polymer chain.

The lower alkyl group for R_8 and Z is, for
example, methyl or ethyl, and examples of the halogen
atom for Z are chlorine and bromine.

30   Examples of such a thermoplastic resin are
polystyrene, a rubber-modified polystyrene (a high-
impact polystyrene), a styrene/butadiene copolymer,
a styrene/butadiene/acrylonitrile copolymer, a styrene/

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acrylic rubber/acrylonitrile copolymer, a styrene/alpha-
methyl-styrene copolymer, and a styrene/butadiene block
copolymer.

At least one such thermoplastic resin can be
used in combination with the polyphenylene ether.

The thermoplastic resin may be included in the
resin matrix in an amount of preferably not more than
95%, especially preferably not more than 80%.

The elastomer which may be used in this
invention is an elastomer in the ordinary sense.
Accordingly, the elastomer in this invention, for example,
includes polymers having a Young's modulus at ordinary
temperature of 10^5 to 10^9 dynes/cm^2 (0.1 to 1020 kg/cm^2),
the Young's modulus being defined at pages 71 to 78 of
A. V. Tobolsky, "Properties and Structures of Polymers"
(John Wiley & Sons, Inc., 1960).

Examples of such an elastomer include poly-
butadiene, polyisoprene, a nitrile rubber, an ethylene/
propylene copolymer, an ethylene/propylene/diene copolymer
(EPDM), polypentenamer, Thiokol rubbers, polysulfide
rubbers, an acrylic rubber, a polyurethane rubber, a grafted
product formed between a butyl rubber and polyethylene,
polyester elastomers, and block copolymers, such as A-B-A'
type block copolymers and A-B'-A' type block copolymers
of diene compounds and vinyl aromatic compounds.

In the above A-B-A' type block copolymers and
A-B'-A' type block copolymers, the terminal blocks A and
A' are polymer chain blocks of the vinyl aromatic compounds.
The central block B in the A-B-A' type block copolymers
is a polymer chain block of a conjugated diene, and the
central block B' in the A-B'-A' type block copolymers is
a block resulting from the hydrogenation of a polymer
chain block of a conjugated diene.

In the above description, the diene, diene
compound and conjugated diene are used in the same sense,
and may, for example, specifically represent 1,3-butadiene,
2,3-dimethylbutadiene, isoprene, 1,3-pentadiene or a mixture

of these. The vinyl aromatic compound may, for example,
denote styrene, alpha-methylstyrene, vinyltoluene, vinyl-
xylene, ethylvinylxylene, vinylnaphthalene, or mixtures
thereof.

5   Preferably, the aforesaid A-B-A' type block
copolymers or A-B'-A' type block copolymers are used as
the elastomer in this invention. The terminal blocks A
and A' of these block copolymers preferably have a number
average molecular weight of about 2,000 to about 100,000,

10   and the central blocks B and B' preferably have a number
average molecular weight of about 25,000 to about 1,000,000.
        The elastomer may be included in the resin
composition of this invention in an amount of preferably
not more than 20% by weight, especially preferably not

15   more than 10% by weight, based on the resin matrix.
        In the polyphenylene ether resin composition
of this invention, the polyphenylene ether may be included
in an amount of at least 5% by weight, preferably at
least 15% by weight, based on the resin matrix.

20   In the polyphenylene ether resin composition of
this invention, the polyphenylene ether resin matrix com-
posed of the polyphenylene ether alone or a mixture of
it with the other polymer contains, dispersed therein,
a phosphonic acid or its derivative represented by the

25   following formula (I)
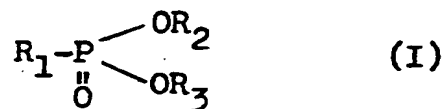
$$R_1 - \underset{\underset{O}{\|}}{P} \underset{\diagdown OR_3}{\overset{\diagup OR_2}{}} \qquad (I)$$

wherein R_1 represents an unsubstituted or
substituted phenyl or naphthyl group, or
a linear or branched alkyl group with 1 to

30   18 carbon atoms, or a group of the following
formula (a)
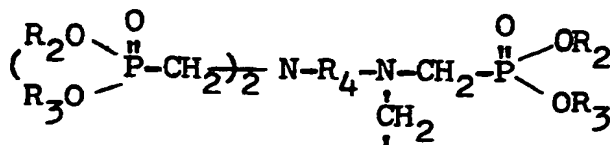
$$\left(\underset{R_3O \diagdown}{\overset{R_2O \diagdown}{}} \underset{}{\overset{\underset{O}{\|}}{P}} - CH_2 \right)_2 N - R_4 - \underset{\underset{CH_2}{|}}{N} - CH_2 - \underset{}{\overset{\underset{O}{\|}}{P}} \underset{\diagdown OR_3}{\overset{\diagup OR_2}{}}$$

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R_2 and R_3 are identical or different and
each represents hydrogen atom s metal, an
unsubstituted or substituted phenyl group,
or an alkyl group with 1 to 10 carbon atoms;

5   and R_4 is a divalent hydrocarbon group with
2 to 10 carbon atoms.

which serves to improve the heat stability of the poly-
phenylene ether.

In formula (I), the substituted phenyl for R_1,
10   R_2 and R_3 or the naphthyl group for R_1 is preferably a
phenyl or naphthyl group which is substituted with 1 to
3 of alkyl groups such as a methyl or ethyl group.
Examples of the unsubstituted or substituted phenyl or
naphthyl groups are preferably phenyl, 4-methylphenyl,
15   2-methylphenyl, 4-ethylphenyl, 2,4-dimethylphenyl, 3,5-
dimethylphenyl, 2,5-dimethylphenyl, 2,4,5-trimethylphenyl,
naphthyl, α-methylnaphthyl, β-methylnaphthyl, α-ethyl-
naphthyl and β-ethylnaphthyl.

The metal for R_1 is preferably an alkali metal
20   such as sodium or alkaline earth metal such as barium.

Examples of the alkyl group with 1 to 18 carbon
atoms for R_1, including examples of the alkyl group with
1 to 10 carbon atoms for R_2 and R_3, which may be linear
or branched, are preferably methyl, ethyl, propyl, butyl,
25   pentyl, hexyl, octyl, nonyl, decyl, dodecyl, tetradecyl,
hexadecyl and octadecyl.

The divalent hydrocarbon group with 2 to 10
carbon atoms for R_4 is preferably an alkylene group with
2 to 10 carbon atoms such as ethylene, 1,2-propylene,
30   1,3-propylene, tetramethylene, pentamethylene, hexamethylene,
heptamethylene, octamethylene, nonamethylene and deca-
methylene.

The phosphonic acids corresponding to general
formula (I) in which R_2 and R_3 are hydrogen include, for
35   example, phenyl phosphonic acid, 4-methylphenyl phosphonic
acid, 2-methylphenyl phosphonic acid, 4-ethylphenyl
phosphonic acid, 2,4-dimethylphenyl phosphonic acid,

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2,5-dimethylphenyl phosphonic acid, 2,4,5-trimethylphenyl
phosphonic acid, 2,4,6-trimethylphenyl phosphonic acid;
naphthyl-1-phosphonic acid, 8-methylnaphthyl-1-phosphonic
acid, 8-methylnaphthyl-2-phosphonic acid, 8-ethylnaphthyl-
5   1-phosphonic acid; methyl phosphonic acid, ethyl phosphonic
acid, propyl phosphonic acid, iso-propyl phosphonic acid,
butyl phosphonic acid, iso-butyl phosphonic acid, amyl
phosphonic acid, iso-amyl phosphonic acid, n-hexyl
phosphonic acid, n-heptyl phosphonic acid, n-octyl
10   phosphonic acid, n-nonyl phosphonic acid, n-decyl phos-
phonic acid, n-dodecyl phosphonic acid, n-tetradecyl
phosphonic acid, n-hexadecyl phosphonic acid, n-octadecyl
phosphonic acid; ethylenediamine tetra(methylene phospho-
nic acid), 1,2-propylenediamine tetra (methylene phosphonic
15   acid), 1,3-propylenediamine tetra(methylene phosphonic
acid), tetramethylenediamine tetra(methylene phosphonic
acid), pentamethylenediamine tetra(methylene phosphonic
acid), hexamethylenediamine tetra(methylene phosphonic
acid), heptamethylenediamine tetra(methylene phosphonic
20   acid), octamethylenediamine tetra(methylene phosphonic
acid), nonamethylenediamine tetra(methylene phosphonic
acid), decamethylenediamine tetra(methylene phosphonic
acid).

Phosphonic acid derivatives (esters) corres-
25   ponding to general formula (I) in which R_2 and/or R_3 are
the unsubstituted or substituted phenyl group or the
alkyl group can be produced by reacting the aforesaid
phosphonic acids with corresponding alcohols or phenols.
Examples of these esters include dimethyl ester, monoethyl
30   ester, diethyl ester, dibutyl ester, di-n-amyl ester,
nonophenyl ester, diphenyl ester or di-p-butylphenyl
ester of phenylphosphonic acid; diethyl ester, diphenyl
ester or di-p-tolyl ester of 4-methyl-phenyl phosphonic
acid; dimethylester, diethyl ester, dipropyl ester,
35   di-iso-propyl ester, diphenyl ester, di-m-tolyl ester
or di-p-tolyl ester of methylphosphonic acid; diethyl
ester or dibutyl ester of ethyl phosphonic acid;

diethyl ester or dipropyl ester of propyl phosphonic
acid; dimethyl ester or dibutyl ester of butyl phosphonic
acid; di-iso-butyl ester of iso-butyl phosphonic acid;
diethyl ester or dibutyl ester of amylphosphonic acid;
5   diethyl ester or diphenyl ester of iso-amyl phosphonic
acid; diethyl ester or dibutyl ester of n-hexyl phosphonic
acid; diethyl ester or dibutyl ester of n-heptyl phosphonic
acid; diethyl ester or dibutyl ester of n-octyl phosphonic
acid; diethyl ester or dibutyl ester of n-nonyl phosphonic
10   acid; diethyl ester or dibutyl ester of n-decyl phosphonic
acid; diethyl ester or dibutyl ester of n-dodecyl phos-
phonic acid; diethyl ester or dibutyl ester of n-tetra-
decyl phosphonic acid; dibutyl ester of n-hexadecyl
phosphonic acid; dibutyl ester of n-octadecyl phosphonic
15   acid; octamethyl ester of ethylenediamine tetra(methylene
phosphonic acid); octamethyl ester of 1,3-propylenediamine
tetra(methylene phosphonic acid); octaethylester of
ethylenediamine tetra(methylene phosphonic acid);
octamethyl ester of hexamethylenediamine tetra(methylene
20   phosphonic acid).

          Examples of phosphonic acid derivatives (salts)
corresponding to general formula (I) in which R_2 and/or
R_3 are a metal such as an alkali metal or an alkaline
earth metal are self-evident and are preferably sodium or
25   barium salts of aforesaid phosphonic acids or half-
esters of phosphonic acids.

          The phosphonic acid or its derivative represented
by general formula (I) may be included in an amount of
about 0.01 to about 10 parts by weight, preferably about
30   0.05 to about 5 parts by weight, especially preferably
about 0.1 to about 3 parts by weight, per 100 parts by
weight of the resin matrix.

          Even when these compounds are used in amounts
exceeding the above upper limits, the heat stability of
35   the resulting resin composition is not correspondingly
improved. Rather, it is frequently deleterious on the
properties of the resin composition, resulting in lowered

heat distortion temperatures, for example. If the amount
of the stabilizer compound is below the specified limit,
the heat stability of the resin composition is not improved
to the expected extent.

5   The resin composition of this invention shows
better heat stability by dispersing both the above phos-
phonic acid or its derivative and at least one sterically
hindered phenol in the matrix resin. It is believed that
the better heat stability is due to the synergistic
10   action of the two kinds of stabilizer compounds.

        Examples of sterically hindered phenols which
can be effectively used in this invention include mono-
hydric phenols such as 2,6-di-tert.-butyl-p-cresol, 2-
tert.-butyl-4-methoxyphenol, 2,4-dinonylphenol, octadecyl-
15   3-(3,5-di-tert.-butyl-4-hydroxyphenyl)propionate, diethyl
3,5-di-tert.-butyl-4-hydroxybenzylphosphonate, and 2-(3',
5'-di-tert.-butyl-4'-hydroxyanilino)-4,6-dioctylthio-
1,3,5-triazine; dihydric phenols such as 2,2'-methylene-
bis(4-methyl-6-tert.-butylphenol),2,2'-methylenebis(4-
20   ethyl-6-tert.-butylphenol), butylidenebis(methyl-butyl-
phenol), 4,4'-thiobis(6-tert.-butyl-3-methylphenol), 1,1-
bis(4-hydroxyphenyl)cyclohexane, 1,6-hexanediol-bis-3-
(3,5-di-tert.-butyl-4-hydroxyphenyl)propionate, 2,2'-
thiodiethyl-bis(3,5-ditert.-butyl-4-hydroxyphenyl)
25   propionate) and N,N'-hexamethylenebis(3,5-di-tert.-butyl-
4-hydroxyhydrocinnamide); trihydric phenols such as 1,3,5-
tris(4-tert.-butyl-3-hydroxy-2,6-dimethylbenzyl)iso-
cyanuric acid, 2,4,6-tris-(3',5'-di-tert.-butyl-4'-
hydroxybenzyl)-1,3,5-triazine, a triester of 3,5-di-tert.-
30   butyl-4-hydroxyhydrocinnamic acid with 1,3,5-tris(2-
hydroxyethyl-S-triazine-2,4,6-(1H,3H,5H)trione) and
1,1,3-tris(2'-methyl-4'-hydroxy-5'-tert.-butylphenyl)
butane; and tetrahydric phenols such as pentaerythrityl-
tetrakis(3-(3,5-di-tert.-butyl-4-hydroxyphenyl)propionate).
35   The sterically hindered phenol may be included
in the resin composition of this invention in an amount
of about 0.05 to about 10 parts by weight, preferably

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about 0.1 to about 5 parts by weight, especially preferably
about 0.5 to about 3 parts by weight, per 100 parts by
weight of the matrix resin.

     The resin composition of this invention shows

5 much better heat stability by dispersing an organic mono-
phosphite or organic polyphosphite as well as the phos-
phonous acid or its ester and the sterically hindered
phenol in the resin matrix.

     Examples of effective organic phosphotes for

10 use in this invention include organic monophosphites such
as triphenyl phosphite, tricresyl phosphite, triisooctyl
phosphite, tridecyl phosphite, tri-2-ethylhexyl phosphite,
trioctadecyl phosphite, tri(octylphenyl)phosphite, tri-
(nonylphenyl)phosphite, tridodecylthio phosphite, phenyl-

15 diethyl phosphite, phenyl-di(2-ethylhexyl)phosphite,
isooctyldiphenyl phosphite, diisooctylmonophenyl phosphite
and di(2-ethylhexyl)mono(isooctylphenyl)phosphite; and
organic polyphosphites such as a phosphite resin of
hydrogenated bisphenol A. Among these organic phosphites,

20 the organic polyphosphites are preferred. An organic
monophosphite may be used in combination with an organic
polyphosphite.

     The organic phosphite may be included into the
resin composition of this invention in an amount of about

25 0.05 to about 10 parts by weight, preferably about 0.1 to
about 5 parts by weight, especially preferably about 0.5
to about 3 parts by weight, per 100 parts by weight of the
resin forming the matrix.

     The resin composition of this invention may

30 further contain various additives depending upon the
intended uses. Examples of the additives include
lubricants, such as olefin waxes typified by polyethylene
wax and polypropylene wax; phosphate-type fire retardants
typified by triphenyl phosphate or tricresyl phosphate;

35 bromine-type fire retardants typified by decabromobiphenyl
pentabromotoluene or decabromobiphenyl ether; pigments
typified by titanium dioxide or zinc oxide; inorganic

fillers typified by glass fibers, asbestos, wollastonite,
mica or talc; and organic fillers typified by carbon
fibers. The amounts of these additives vary depending
upon their types, but should be within the ranges which
5   do not degrade the heat stability of the resin composition
of this invention.

The resin composition of this invention can be
easily produced by melt-mixing methods known with regard
to thermoplastic resins. For example, it can be prepared
10   conveniently by a method which comprises mixing the poly-
phenylene ether or a mixture of it with another polymer
such as a thermoplastic resin or elastomer, with prede-
termined amounts of the phosphonous acid or its ester,
and optionally the sterically hindered phenol and optional-
15   ly the organic phosphite in a mixer, then kneading the
mixture fully in a melt-extruder, and pelletizing the
resulting homogeneous molten mixture.

The following Examples and Comparative Examples
illustrate the resin composition of this invention more
20   specifically. Unless otherwise specified, all parts and
percentages in these examples are by weight.

Example 1 and Comparative Example 1

Sixty (60) parts of a 2,6-dimethylphenol/2,3,6-
trimethylphenol copolymer (2,3,6-trimethylphenol 5 mole%)
25   having an intrinsic viscosity, measured at 25°C using
chloroform as a solvent, of 0.52 dl/g, 37 parts of high-
impact polystyrene (the polystyrene matrix having an
intrinsic viscosity, measured at 25°C using chloroform
as a solvent, of 0.89 dl/g; gel content analyzed by
30   using a mixture of methyl ethylketone and acetone as a
solvent of 12.9% by weight), 2 parts of a polystyrene/
polybutadiene/polystyrene block copolymer (the weight
ratio of the polystyrene blocks to the polybutadiene
block 30:70; the viscosity of a 20% toluene solution of
35   the copolymer measured at 25°C using a Brookfield model
RVT viscometer, of 1500 cps), 1 part of an ethylene/
propylene copolymer (having a reduced specific viscosity,

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measured at $135^{\circ}C$ in a concentration of 0.1 g/100 ml
using decalin as a solvent, of 2.0 and a glass transi-
tion point of $-49^{\circ}C$), 5.8 parts of triphenyl phosphate,
7 parts of titanium dioxide and 1.0 part of phenylphos-

5   phonic acid $\left(C_6H_5P(OH)_2\right)$ were fully mixed in a Henschel
         $\overset{\parallel}{O}$

mixer. The resulting mixture was pelletized by a twin-
screw extruder (AS-30, a product of Nakatani Kikai
Seisakusho) in which the maximum temperature of the
cylinder was set at $290^{\circ}C$. A test specimen, 1/8 inch

10   thick, for measurement of Izod impact strength was
molded from the resulting pellets under an injection
pressure of 1050 kg/cm^2 using an injection molding machine
(SJ-35B, a product of Meiki Seisakusho). The test
specimen was aged in hot air at $115^{\circ}C$ for 10 days.

15   Its Izod impact strength was measured before and after
the aging. The results are tabulated below.

        For comparison, the above procedure was repeated
except that the phenylphosphonic acid was not used. The
Izod impact strength of the test specimen not containing

20   the phosphonic acid was measured and the results are also
tabulated below (Comparative Example 1).

	Izod impact strength (notched, kg-cm/cm)	
	Before aging	After aging
Example 1	24.5	17.6 (72%)
Comparative Example 1	19.6	11.4 (58%)

        In the above and subsequent tables, the
parenthesized figures show the percent retention calcu-

25   lated as follows:

$$\text{Retention (\%)} = \frac{\text{Izod impact strength after aging}}{\text{Izod impact strength before aging}} \times 100$$

        The above table clearly shows that the use of
phenylphosphonic acid improved the Izod impact strength
of the molded product after the aging.

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<u>Example 2</u>

The procedure of Example 1 was repeated except that 1.0 part of diethyl phenylphosphonate $((C_6H_5P(OC_2H_5)_2)$ with O beneath the P)

was used instead of 1.0 part of phenylphosphonic acid.
5   The test specimen was examined for Izod impact strength in the same way as in Example 1.

The results of Example 2 are tabulated below together with the result of Comparative Example 1.

	Izod impact strength (notched, kg-cm/cm)	
	<u>Before aging</u>	<u>After aging</u>
Example 2	24.4	19.2 (79%)
Comparative Example 1	19.6	11.4 (58%)

10   <u>Example 3</u>

The pellets produced in Example 2 were left to stand for 20 minutes in the molten state in the cylinder of an injection molding machine (SJ-35B) in which the maximum temperature of the cylinder was set at $280^{\circ}C$,
15   and thereafter injection-molded to prepare test specimens for measurement of Izod impact strength. The results are tabulated below together with the data obtained in Example 2.

	Izod impact strength (notched, kg-cm/cm)	
	Method of <u>Example 1</u>	<u>After 20 minute standing</u>
Example 3	24.4	20.3 (83%)

20   It is seen from the above table that the resin composition of this invention shows a high retention of Izod impact strength even after it has been subjected to a heat history at high temperature.

<u>Example 4 and Comparative Example 2</u>
25   The procedure of Example 1 was repeated except that 0.4 part of phenyl phosphonic acid and 0.6 part of

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2,2'-methylene bis(4-methyl-6-tert-butylphenol) were
used instead of 1.0 part of phenylphosphonic acid. The
test specimen was examined for Izod impact strength in
the same way as in Example 1.

5   For comparison, the results of Comparative
Example 2 wherein only 1 part of 2,2'-methylene bis(4-
methyl-6-tert-butylphenol) was used are tabulated below
together with the results of Example 4.

	Izod impact strength (notched, kg-cm/cm)	
	Before aging	After aging
Example 4	23.9	17.9 (75%)
Comparative Example 2	19.3	10.6 (55%)

10   Example 5 and Comparative Example 3

          The procedure in Example 3 was repeated except
that 0.4 part of diethyl phenylphosphonate and 0.6 part
of 2,2'-methylene bis(4-methyl-6-tert-butylphenol) were
used instead of 1.0 part of diethyl phenylphosphonate.
15   The test specimen was examined for Izod impact strength
in the same way in Example 3.

          For comparison, the results of Comparative
Example 3 wherein only 1 part of 2,2'-methylene bis(4-
methyl-6-tert-butylphenol) was used are tabulated below
20   together with the results of Example 5.

	Izod impact strength (notched, kg-cm/cm)	
	Method of Example 1	After 20 minute standing
Example 5	23.7	20.7 (87%)
Comparative Example 3	19.3	14.6 (76%)

Example 6 and Comparative Example 4

          59 Parts of poly(2,6-dimethyl-1,4-phenylene)
ether having an intrinsic viscosity, measured at 25°C in
25   chloroform, of 0.54 d./g, 59 parts of the same high-impact

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polystyrene as used in Example 1, 2 parts of the same polystyrene/polybutadiene/polystyrene block copolymer as used in Example 1, 10.5 parts of triphenyl phosphate, 7 parts of titanium dioxide, 1 part of n-octyl phosphonic

5   acid were fully mixed in a Henschel mixer. The mixture was pelletized by a twin-screw extruder (AS-30) in which the maximum temperature of the cylinders was set at 290°C. The pellets were injection-molded under an injection pressure of 1050 kg/cm^2 using an injection molding machine

10 (SJ-35B) to prepare a test specimen, 1/8 inch thick, for measurement of Izod impact strength. The resulting test specimen was aged in hot air at 85°C for 20 days, and its Izod impact strength was measured before and after the aging. The results are tabulated below.

15   For comparison, the above procedure was repeated except that the n-octylphosphonic acid was not used. The results are also tabulated below (Comparative Example 4).

	Izod impact strength (notched; kg-cm/cm)	
	Before aging	After aging
Example 6	11.4	9.7 (85%)
Comparative Example 4	10.2	7.6 (75%)

20 <u>Example 7 and Comparative Example 5</u>

        85 Parts of the same 2,6-dimethylphenol/2,3,6-trimethylphenol copolymer as used in Example 1, 15 parts of the same high-impact polystyrene as used in Example 1, 5 parts of titanium dioxide, 0.2 part of diphenyl 4-

25 methylphenylphosphonate and 1.8 parts of 2,6-di-tert-butyl-p-cresol were fully mixed in a Henschel mixer. The resulting mixture was pelletized by a twin-screw extruder (AS-30) in which the maximum temperature of the cylinders was set at 300°C. The pellets were injection-

30 molded under an injection pressure of 1320 kg/cm^2 by an injection molding machine (SJ-35B) in which the maximum temperature of the cylinder was set at 320°C. to prepare

a test specimen, 1/8 inch thick, for measurement of Izod impact strength. The test specimen was aged in hot air at 120°C for 100 hours. The Izod impact strengths of the specimen was measured before and after the aging, and the
5   results are tabulated below.

For comparison, the above procedure was repeated except that the stabilizer compounds were not added. The results are also shown in the following table (Comparative Example 5).

	Izod impact strength (notched, kg-cm/cm)	
	Before aging	After aging
Example 7	8.5	7.2 (85%)
Comparative Example 5	8.8	5.3 (60%)

Examples 8 to 10 and Comparative Example 6

Ten parts of the same 2,6-dimethylphenol/2,3,6-trimothylphenol copolymer used as in Example 1 was added to a solution of 0.1 part of diethyl phenylphosphonate in
15   a suitable amount of acetone. The mixture was slurried by agitation. It was dried in the air overnight and then further dried at 40°C under reduced pressure for 8 hours (Example 8). The above procedure was repeated except that the amount of the diethyl phenylphosphonate was
20   changed to 0.2 part (Example 9) and 0.4 part (Example 10).

1.5 Parts of each of the powdery mixtures was molded into cylindrical specimens under pressure. The test specimens were set in a heater of a Koka-type flow tester (a product of Shimadzu Seisakusho) maintained at
25   280°C, and allowed to stand for 60 minutes under a pressure of 30 kg/cm^2. Sixty minutes later, the heater was fully cooled, and the Hunter's whitenesses of the cylindrical specimens were measured by a color difference meter (Color Studio CS-K5F, a product of Nippon Denshoku
30   Kogyo K.K.). The results were tabulated below.

For comparison, test specimens were prepared in accordance with the procedure under the same conditions as above except that diethyl phenylphosphonate was not

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added. The results are shown in the following table
(Comparative Example 6).

	Hunter's whiteness
Example 8	20.0
Example 9	21.2
Example 10	21.3
Comparative Example 6	14.9

5   The results in the above table show that the
diphosphonite is effective for inhibiting coloration of
the polyphenylene ether resin under heat.

Example 11 and Comparative Example 7

        An autoclave was charged with 100 parts of
ethylbenzene and 165 parts of poly(2,6-dimethyl-1,4-
10   phenylene)ether having an intrinsic viscosity, measured
at 25°C in chloroform, of 0.55 dl/g, and they were heated
to 125°C with stirring to form a solution. Then, 7
parts of di-tert.-butyl peroxide and 65 parts of styrene
were added to the solution and reacted at 165°C for 2
15   hours. The reaction mixture was withdrawn, and dried at
215°C under reduced pressure for 2 hours to remove
ethylbenzene and the unreacted styrene to afford styrene-
grafted poly(2,6-dimethyl-1,4-phenylene)ether. The grafted
copolymer had a polystyrene content of about 25%.

20   The procedure in Example 8 was repeated except
that 10 parts of the grafted copolymer and ethanol were
used instead of 10 parts of 2,6-dimethylphenol/2,3,6-
trimethylphenol copolymer and acetone. The resulting
cylindrical specimens were examined for Hunter's whiteness.
25   The results are tabulated below.

        For comparison, the result from a cylindrical
specimen prepared from the grafted copolymer is also
tabulated below.

	Hunter's whiteness
Example 11	15.7
Comparative Example 7	13.8

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<u>Example 12 and Comparative Example 8</u>

The procedure in Example 1 was repeated except that 1 part of 1,2-propylenediamine tetra(methylene phosphonic acid) was used instead of 1.0 part of phenyl-
5 phosphonic acid. The test specimen was examined for Tensile impact strength.

For comparison, the results of Comparative Example 8 wherein 1,2-propylenediamine tetra(methylene phosphonic acid) was not used are also tabulated below.

	Tensile impact strength $(kg/cm/cm^2)$	
	Before aging	After aging
Example 12	140	111 (79%)
Comparative Example 8	178	71 (40%)

In the above and subsequent tables, the parenthesized figures show the percent retention calculated as follows:

$$\text{Retention (\%)} = \frac{\text{Tensile impact strength after aging}}{\text{tensile impact strength before aging}} \times 100$$

15 The above table clearly shows that the use of 1,2-propylenediamine tetra(methylene phosphonic acid) improved the tensile impact strength of the molded product after the aging.

<u>Example 13</u>

20 The procedure in Example 12 was repeated except that 0.4 part of 1,2-propylenediamine tetra-(methylene phosphonic acid) and 0.6 part of 2,2'-methylene bis(4-methyl-6-tert-butylphenol) were used instead of 1 part of 1,2-propylenediamine tetra(methylene
25 phosphonic acid). The results of Example 13 are tabulated below.

	Tensile impact strength $(kg.cm/cm^2)$	
	Before aging	After aging
Example 13	173	116 (67%)

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Example 14 and Comparative Example 9

The forepart of the procedure in Example 6 was repeated except that 1 part of hexamethylenediamine tetra(methylene phosphonic acid) was used instead of 1 part of n-octyl phosphonic acid.

The resulting pellets were injection-molded under an injection pressure of 1050 kg/cm^2 using an injection molding machine (SJ-35B) in which the maximum temperature of the cylinders was set at 290°C to prepare a test specimen, 1/16 inch thick, S-type, for measurement of tensile impact strength.

The resulting test specimen was aged in hot air at 85°C for 20 days, and Tensile impact strength was measured before and after the aging. The results are tabulated below.

For comparison, the above procedure was repeated except that the hexamethylenediamine tetra(methylene phosphonic acid) was not used, the results are also tabulated below.

	Tensile impact strength (kg.cm/cm^2)	
	Before aging	After aging
Example 14	150	105 (70%)
Comparative Example 9	145	70 (48%)

Example 15

The procedure in Example 1 was repeated except that 2 parts of 1,2-propylenediamine tetra(methylene phosphonic acid) was used. The results are tabulated below.

	Tensile impact strength (kg.cm/cm^2)	
	Before aging	After aging
Example 15	144	118 (82%)

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Example 16 and Comparative Example 9

The forepart of the procedure in Example 1 was repeated except that 1 part of di-sodium salt of phenyl-phosphonic acid was used instead of 1 part of phenyl-

5   phosphonic acid.

A test specimen, 1/16 inch, S-type, for measure-ment of tensile impact strength was molded from the resulting pellets under an injection pressure of 1050 kg/cm^2 using an injection molding machine (SJ-35B) in which

10 the maximum temperature of the cylinder was set at $280^\circ C$. The test specimen was aged in hot air at $115^\circ C$ for 10 days. The tensile impact strength was measured before and after the aging. The results are tabulated below.

For comparison, the above procedure was repeated

15 except that the disodium salt of phenylphosphonic acid was not used. The results was also tabulated below.

	Tensile impact strength $(kg.cm/cm^2)$	
	Before aging	After aging
Example 16	159	102 (64%)
Comparative Example 9	178	71 (40%)

The above table clearly shows that the use of di-sodium salt of phenylphosphonic acid improved of the

20 molded product after the aging.

Example 17 and Comparative Example 10

The procedure in Example 16 was repeated except that 0.4 part of di-sodium salt of phenylphosphoric acid and 0.6 part of 2,2'-methylene bis(4-methyl-6-

25 tert-butylphenol) were used instead of 1 part of di-sodium salt of phenylphosphonic acid. The results are tabulated below together with the results of Example 16.

For comparison, the procedure in Example 16 was also repeated except that 1 part of 2,2-methylene

30 bis(4-methyl-6-tert-butylphenol) was used instead of 1 part of disodium salt of phenylphosphonic acid. The

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results are also tabulated below.

The table clearly shows a synergistic effect between disodium salt of phenylphosphonic acid and the hindered phenolic compound.

Example No.	Additives	Tensile impact strength (kg-cm/cm^2)	
		Before aging	After aging
17	disodium salt of phenylphosphonic acid 0.4 part and 2,2'-methylene bis(4-methyl-6-tert-butylphenol) 0.6 part	131	121 (92%)
16	di-sodium salt of phenylphosphonic acid 1.0 part	159	102 (64%)
Comparative Example 10	2,2'-methylene bis(4-methyl-6-tert-butylphenol) 1.0 part	132	77 (58%)

Example 18 and Comparative Example 11

The procedure in Example 14 was repeated except that 1 part of barium salt of phenylphosphonic acid was used instead of 1 part of hexamethylenediamine tetra-(methylene phosphonic acid).

The resulting test specimen was aged in hot air at 85°C for 20 days, and tensile impact strength was measured before and after the aging. The results are tabulated below.

For comparison, the above procedure was repeated except that the barium salt of phenylphosphonic acid was not used. The results are also tabulated below.

	Tensile impact strength (kg.cm/cm^2)	
	Before aging	After ageng
Example 18	153	110 (72%)
Comparative Example 11	145	70 (48%)

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<u>Example 19</u>

The procedure in Example 16 was repeated except
that 2.0 part of di-sodium salt of phenylphosphonic acid
was used. The results was tabulated below.

$$\text{Tensile impact strength}$$
$$\underline{(\text{kg.cm/cm}^2)}$$

	Before aging	After aging
Example 19	150	102 (68%)

<u>Example 20 and Comparative Example 12</u>

The procedure in Example 16 was repeated except
that 0.4 part of di-sodium salt of phenylphosphonic acid
and 0.6 part of 2-(3',5'-di-tert-butyl-4'-hydroxyanilino)-
4,6-di-octylthio-1,3,5-triazine ("Irganox 565"[R], product
of Ciba Geigy Co.) were used instead of 1.0 part of di-
sodium salt of phenylphosphonic acid. The results are
tabulated below.

For comparison, the procedure in Example 16
was also repeated except that 1 part of "Irganox565"[R]
was used instead of 1 part of di-sodium salt of phenyl-
phosphonic acid. The results are also tabulated below
together with the results of Fxample 16.

Example No.	Additive	Tensile impact strength (kg.cm/cm^2)	
		Before aging	After aging
20	di-sodium salt of phenylphosphonic acid 0.4 part and Irganox 565[R] 0.6 part	144	108 (75%)
16	di-sodium salt of phenylphosphonic acid 1.0 part	159	102 (64%)
Comparative Example 12	Irganox 565[R]	140	82 (59%)

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C L A I M S

1.   A polyphenylene ether resin composition having
improved heat stability and comprising a polyphenylene ether
resin matrix having dispersed therein a phosphorus compound
as stabilizer, characterised in that the stabilizer is a
phosphonic acid or a derivative thereof of the formula
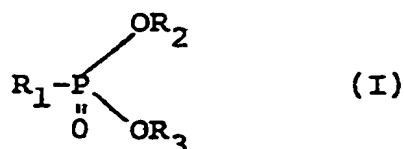
$$R_1-\underset{\underset{O}{\|}}{P}\overset{OR_2}{\underset{OR_3}{<}} \qquad (I)$$

wherein R_1 represents an unsubstituted or substituted phenyl
or naphthyl group, or a linear or branched alkyl group of 1
to 18 carbon atoms, or a group of the formula (a)

$$(\overset{R_2O}{\underset{R_3O}{>}}\underset{\underset{}{\|}}{P}-CH_2\overline{\smash{)}}_2N-R_4-\underset{\underset{CH_2-}{|}}{N}-CH_2-\underset{\underset{O}{\|}}{P}\overset{OR_2}{\underset{OR_3}{<}} \quad ;$$
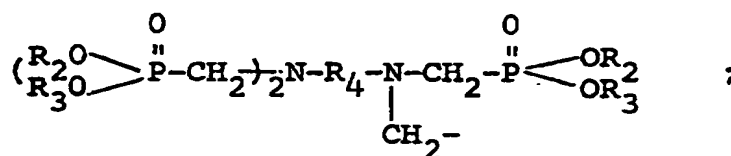
R_2 and R_3 are the same or different and each represents
hydrogen atom, a metal, an unsubstituted or substituted
·phenyl group, or an alkyl group of 1 to 10 carbon atoms
and R_4 is a divalent hydrocarbon group of 2 to 10 carbon
atoms.

2.   A composition according to claim 1 which also
comprises at least one sterically hindered phenol dispersed
in the polyphenylene ether resin matrix.

3.   A composition according to claim 1 or 2
which also comprises at least one organic monophosphite
or polyphosphite dispersed in the polyphenylene ether
resin matrix.

4.   A composition according to claim 1, 2 or 3 wherein
the polyphenylene ether resin constituting the matrix is a
polyphenylene ether homopolymer or copolymer obtained by
polycondensing at least one mononuclear phenol of the formula

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(III)
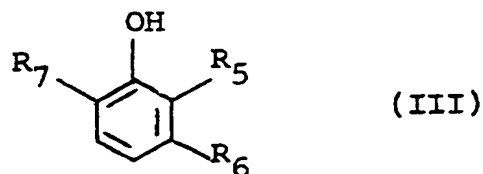
wherein R_5, R_6 and R_7, which may be the same
or different, each represent a hydrogen atom
or an alkyl group of 1 to 3 carbon atoms,
provided that at least one or R_5 and R_7 is an
alkyl group of 1 to 3 carbon atoms,

or a grafted polyphenylene ether obtained by graft-
copolymerizing said polyphenylene ether with a vinyl
aromatic compound.

5.   A composition according to claim 4 wherein the
vinyl aromatic compound is selected from styrene,
alpha-methylstyrene, vinyltoluene and vinylxylene.

6.   A composition according to claim 1, 2 or 3
wherein the polyphenylene ether resin constituting the
matrix is poly(2,6-dimethyl-1,4-phenylene)ether, a
polyphenylene ether copolymer derived from 2,6-dimethyl-
phenol and 2,3,6-trimethylphenol, or a grafted poly-
phenylene ether obtained by grafting styrene to any
such polyphenylene ether.

7.   A composition according to any one of the
preceding claims wherein the polyphenylene ether resin
matrix comprises a mixture of a polyphenylene ether with
another polymer.

8.   A composition according to claim 7 wherein said
other polymer is a thermoplastic resin containing at least
25% by weight of a recurring structural unit of the
formula

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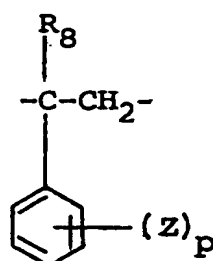
$$
\begin{array}{c}
R_8 \\
| \\
-C-CH_2- \\
| \\
\end{array}
$$
(with substituted phenyl ring)—$(Z)_p$

(II)

     wherein R_8 represents a hydrogen atom or a lower
     alkyl group, Z represents a halogen atom or a
     lower alkyl group, and p is 0 or an integer of
     1 to 3,

in the polymer chain.

9.   A composition according to claim 7 wherein said
other polymer is an elastomer having a Young's modulus
at ordinary temperature of 10^5 to 10^9 dynes/cm^2.

10.   A composition according to claim 9 wherein the
elastomer is an elastomeric block copolymer of the type
A-B-A' wherein A and A' each represent a polymer chain
block of a vinyl aromatic compound and B represents a
polymer chain block of a conjugated diene, or of the type
A-B'-A' wherein A and A' are as defined above, and B'
represents a hydrogenated polymer chain block of a
conjugated diene.

11.   A composition according to claim 10 wherein the
vinyl aromatic compound is selected from styrene, alpha-
methylstyrene, vinyltoluene, vinylxylene, ethylvinylxylene,
vinylnaphthalene and mixtures of two or more of these.

12.   A composition according to claim 10 or 11 wherein the
conjugated diene is selected from 1,3-butadiene, 2,3-dimethyl-
butadiene, isoprene, 1,3-pentadiene and mixtures of two or more
of these.

13. .   A composition according to any one of claims 7 to 12
wherein the amount of the polyphenylene ether is at least
5% by weight of the resin matrix.

14.   A composition according to any one of the preceding
claims wherein R_1, R_2 or R_3 is phenyl substituted with 1 to

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3 alkyl groups.

15.   A composition according to any one of claims 1 to 13 wherein R_1 is naphthyl substituted with 1 to 3 alkyl groups.

16.   A composition according to any one of the preceding claims wherein R_2 or R_3 is an alkali or alkaline earth metal.

17.   A composition according to claim 16 wherein the alkali metal is sodium or the alkaline earth metal is barium.

18.   A composition according to any one of the preceding claims wherein R_4 is an alkylene group of 2 to 10 carbon atoms.

19.   A composition according to any one of the preceding claims wherein the amount of the phosphonic acid or derivative is 0.01 to 10 parts by weight per 100 parts by weight of the polyphenylene ether resin matrix.

20.   A composition according to claim 2 wherein the amount of the sterically hindered phenol is 0.05 to 10 parts by weight per 100 parts by weight of the polyphenylene ether resin matrix.

21.   A composition according to claim 3 wherein the amount of theorganic monophosphite and/or organic polyphosphite is 0.05 to 10 parts by weight per 100 parts by weight of the polyphenylene ether resin matrix.

22.   A shaped article of a resin composition as claimed in any one of the preceding claims.

European Patent Office

EUROPEAN SEARCH REPORT

0038183

EP 81 30 1564

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
X	<u>US - A - 4 076 690</u> (S. ROSENBERGER) * Claim 1; column 4, line 68 * --	1
X	<u>FR - A - 2 244 772</u> (CIBA-GEIGY A.G.) * Claim 1; page 7, line 30 * --	1
A	<u>FR - A - 1 577 890</u> (SUMITOMO) * Claims * --	2-4,6, 20,21
A	<u>FR - A - 2 075 963</u> (SUMITOMO) * Claims * --	2-5, 19-22
A	<u>FR - A - 2 027 757</u> (GENERAL ELEC- TRIC COMP.) * Claims * ----	7,8, 10-12

CLASSIFICATION OF THE APPLICATION (Int. Cl.³)

C 08 L 71/04
        51/08
        53/02

TECHNICAL FIELDS SEARCHED (Int. Cl.³)

C 08 L 71/04
        51/08
        53/02
C 08 K 5/53

CATEGORY OF CITED DOCUMENTS

X: particularly relevant
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E: conflicting application
D: document cited in the application
L: citation for other reasons

&: member of the same patent family, corresponding document

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	21-07-1981	HOFFMANN

EPO Form 1503.1 06.78

Recombinant Lymphokines and Their Receptors

edited by

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MARCEL DEKKER, INC.　　　New York and Basel

Series Introduction

It has been obvious for many years that the cells of the immune system
communicate with one another on a continuing basis. How else could such
an intricate system be kept in proper balance? And how could it respond
to so many different stimuli in a stereotyped pattern? For a time, it
appeared that most regulatory interactions required cell-to-cell contact.
Gradually, however, evidence has accumulated that soluble products are
also intricate members of the immunological community.

One by one, these soluble products have been isolated and their individ-
ual functions defined. The receptor(s) for each, and its target cell popula-
tion, have been determined. The latest and potentially most exciting phase
of investigation has begun with the production of some of these regulatory
substances—the interleukins and colony-stimulating factors, for instance—by
recombinant cDNA cloning. The availability of these substances in large
quantities now opens new, exciting possibilities for treatment of infectious,
immunological, and malignant diseases. In the present volume, Dr. Gillis
and his collaborators have given us a comprehensive and authoritative
glimpse of that future.

Noel R. Rose

9

cDNA Cloning, Expression, and Activity of Human Granulocyte-Macrophage Colony-Stimulating Factor

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that is required for the growth and differentiation of hematopoietic progenitor cells into granulocytes and macrophages (1–3). There has been great interest in studying this protein both to determine more about its biological function and to ascertain its possible value as a therapeutic agent for antitumor therapy and control of immune system deficiencies.

The difficulty of isolating sufficient quantities of purified GM-CSF has led to interest in isolating the GM-CSF gene. Our group and others have now isolated and expressed cDNAs encoding GM-CSF (4–6). Use of recombinant GM-CSF (rGM-CSF) has aided in determining that this protein has neutrophil migration inhibition factor (NIF-T) activity (7), that in the presence of erythropoietin 1 it has burst-promoting activity for erythroid burst-forming units, and that it can stimulate the formation of multipotent colonies containing granulocytes, monocytes, erythroid cells, and megakaryocytes (8).

We here discuss the strategies we have used for isolation of the gene and its expression in yeast and *Escherichia coli*. Additionally, we describe a

new activity of recombinant GM-CSF, namely its ability to stimulate macrophage-mediated killing of tumor cells.

MATERIALS AND METHODS

Construction of Yeast Expression Plasmids

A DNA fragment containing the coding sequence of mature GM-CSF was obtained by digesting (pHG23) (Fig. 1 and Ref. 4) with SfaNI, which cleaves near the mature NH_2-terminus, and NcoI, which cleaves in the 3' noncoding region of the cDNA. This fragment was then engineered into the expression vectors (pαADH2HuGM) and (pYαfHuGM) (Fig. 2), designed for the synthesis and secretion of GM-CSF in yeast. All restriction enzyme, polymerase, and ligase reactions were performed according to manufacturer's procedures. DNA fragment purifications (ELUTIP-Schleicher and Schuell) and E. coli transformations were performed according to established procedures (9).

These vectors contain DNA sequences from (pBR322) for selection and replication in E. coli (the Ap^r gene and origin of replication) and yeast (the TRP-1 gene and 2-μ origin of replication). Expression of foreign genes is under control of the glucose repressible ADH2 promoter (10,11), obtained through the Washington Research Foundation, or the α-factor promoter (12,13). Adjacent to the promoter in both plasmids is the α-factor leader sequence sufficient to direct the secretion of heterologous proteins (13,14). The α-factor leader sequence had been modified by the addition of a second KEX2-processing site to allow complete processing of secreted protein (13). The SfaNI/NcoI human GM-CSF fragment was inserted in-frame with the α-factor leader peptide by means of a synthetic oligonucleotide recreating the amino-terminal coding region to the SfaNI site.

Growth of Yeast Strains

Saccharomyces cerevisiae strain 79 (α, trp1, leu2) was grown in either selective medium [YNB–trp, consisting of 0.67% yeast nitrogen base (Difco), 0.5% casamino acids, 2% glucose, 10 μg/ml adenine, and 20 μg/ml uracil] or rich medium (YPD, consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil). Yeast transformations were performed as described by Hinnen (15) selecting for Trp⁺ transformants.

(a)

PsI   PsI   SfaNI   PsoI   NcoI   SspI

pHG23

pHG25

100bp

(b)

Figure 1 (a) Partial restriction map of human GM-CSF cDNA. Coding sequences are boxed: the open box represents the signal sequence and the shaded box represents the coding region for mature protein. The sizes of the cDNA inserts in (pHG23) and (pHG25) are indicated. (b) The nucleotide sequence and the predicted amino acid sequence for human GM-CSF. The cDNA sequence of the complete 5' and 3' untranslated regions is not shown. The nucleotides are numbered from the presumed initiator methionine codon. The amino acids are numbered from the amino-terminus of the mature protein (star; Ref. 5). Triangles indicate possible N-linked glycosylation sites.

Apr

HindIII

pαADH2HuGM
or
pYαHuGM

TRPI

2μ

EcoRI

Yeast promoter: ADH2 or α-factor

ATG

α-lacto leader

HindIII (SfaNI)

NcoII

HindIII

ACCT TCT TTG GAT AAA AGA GC
    AGA AAC CTA TTT TCT CGT GGG
    ser leu asp lys arg ala ala pro

α-factor leader

HindIII

α-factor processing

Figure 2 Structure of the yeast expression vectors (pαADH2HuGM) and (pYαHuGM). The yeast-*E. coli* shuttle vector contains sequences from (pBR322) that allow selection (Ap^r) and replication in *E. coli* (thick lines), the yeast *TRP*-1 gene, and the 2 μ yeast origin of replication for selection and autonomous replication in a *trp1* yeast strain (thin lines). The small EcoRI to HindIII fragment includes the yeast α-factor promoter adjacent to the α-factor leader sequence, or alternatively, the ADH2 promoter adjacent to the α-factor leader. The synthetic oligonucleotides shown are used to fuse the α-factor sequences with GM-CSF sequences (hatched box). Restriction sites shown in parentheses were lost during construction.

Cultures were prepared for biological assay by inoculating 20-50 ml of rich medium with the appropriate strains and growing them at 30°C to the stationary phase. Cells were then removed by centrifugation, the medium was filtered through a 0.45 μm cellulose acetate filter, and phenylmethyl-sulfonyl fluoride was added (to 1 mM) to control proteolysis. Sterile supernatants were stored at 4°C.

Larger-scale fermentations were done in a 10 L New Brunswick Micro-ferm fermentor. Cells were removed from the medium using a Millipore Pellicon filtration system.

Construction of *Escherichia coli* Expression Plasmids

A 525-bp fragment containing the GM-CSF-coding region was produced by NcoI digestion of (pHG23) followed by blunting with T4 polymerase and digestion with SfaNI. A vector fragment from (pLNIL2) (16) was produced by digestion with XbaI and StuI. This vector fragment was ligated with the GM-CSF-coding fragment and with synthetic oligonucleotides that have XbaI and SfaNI-compatible ends and that recreate the translation initiation sequence of (pLNIL2) and the first two codons of GM-CSF (Fig. 3). The resultant plasmid, (pLNGM), was transformed into *E. coli* strain RR1 (ATCC No. 31343) harboring the plasmid (pRK248cIts) (ATCC No. 33766, Ref. 17), a plasmid that is compatible for coexistence with (pLNGM) in *E. coli* and contains the bacteriophage lambda cI857 tempera-ture-sensitive repressor of PL. (pLNGM) was transferred into strain MM294 (pRK248cIts), which is *hsdR*⁻, *hsdM*⁺ (ATCC No. 33625) prior to transfer into CAG629(pACYC184cIts), which is *hsdR*⁺ *hsdM*⁺. CAG629 (a gen-erous gift from Carol A. Gross, University of Wisconsin) is a derivative of *E. coli* strain SC122 (18) and has the genotype *lacZam trpam phoam supCts malrpsL phe rel lon⁻ htpR⁻ tef*. (pACYC184cIts) was constructed by inserting the 2.4-kb BglII DNA fragment containing the cI857 gene from bacteriophage lambda into (pACYC184) at the BamHI site (19).

The construction of plasmids (pLB5001) and (pLB5001-4) is described in detail elsewhere (Libby et al., manuscript in preparation). Plasmid (pIN-III-ompA-3) (20) was received from Masayori Inouye (SUNY, Stony Brook). A fragment from (pIN-III-ompA-3) was generated by BamHI digestion followed by treatment with AMV reverse transcriptase (Boehringer Mann-heim) and EcoRI digestion. The vector fragment was ligated to the GM-CSF-coding region generated by SfaNI and SspI digestion of (pHG23), and to short synthetic oligonucleotides bearing EcoRI and SfaNI cohesive ends. The JM107 transformants containing the resultant plasmid, (pLB5001), expressed human GM-CSF with four additional amino-terminal amino acids. (pLB5001-4) (Fig. 4) producing the mature form of GM-CSF beginning with alanine-1 (see Fig. 1) was generated from (pLB5001) by in vitro mutagenesis (21) using an oligonucleotide 24 residues in length directing the deletion of 12 bp at the junction of *ompA* and GM-CSF-coding se-quences. The nucleotide sequence in this region of (pLB5001-4) was confirmed using dideoxy sequencing (22).

The plasmid (pLNompGM) (see Fig. 3) was constructed by using the 625-bp fragment obtained from (pLB5001-4) by NcoI digestion, blunting

Figure 3 Structure of *E. coli* expression vectors using the P_L promoter. The plasmids differ only in the region between the P_L promoter and the transcription terminator (t11). The ampicillin resistance gene (Ap^r) and the origin of replication (*ori*) are derived from (pBR322). SD denotes the Shine-Dalgarno sequences. The alanine listed at the end of each oligo-nucleotide is the first alanine of mature GM-CSF. The dotted line in the (pLNompGM) oligonucleotide represents 51 nucleotides encoding the internal portion of the *ompA* signal (20).

with the Klenow fragment of *E. coli* polymerase I (Boehringer Mannheim) and XbaI digestion. This fragment was ligated to the XbaI and StuI-digested (pLNIL2) vector fragment described previously to produce (pLNompGM).

Expression of rGM-CSF in *E. coli*

The strains RR1(pRK248cIts) or CAG629(pACIC84cIts) containing (pLNGM) were propagated in LB (10 g yeast extract, 5 g tryptone, 5 g NaCl) or superinduction medium (SIM; 23) at 30°C using ampicillin, and tetracycline or chloramphenicol, respectively (9). Overnight cultures grown in SIM plus antibiotics were diluted 100-fold into the same medium and vigorously shaken until the A_600 was 0.4-0.5.

Figure 4 Structure of the *E. coli* expression vector (pLB5001-4). This plasmid is identical with the (pIN-III-ompA-3) vector (20), except for the region downstream of the *ompA* signal (clear box) that contains GM-CSF cDNA (hatched box). Abbreviations: ampicillin resistance (Ap^r), origin of replication (*ori*), hybrid *lpp* promoter and *lac* promoter-operator (lpp^p/lac^{PO}), *lpp* transcriptional terminator (lpp^{ter}) and *lac* repressor gene (*lacI*).

The cultures were then induced by temperature shift to 42°C and 1-ml samples were removed at various times, centrifuged, and frozen as previously described (24). Cell pellets were resuspended for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunological analysis in SDS sample buffer containing β-mercaptoethanol (25) and visualized by either silver (26) or Coomassie blue R-250 staining (25). Duplicate cell pellets to be tested for biological activity were resuspended in 30 mM Tris (pH 7.5), 10 mM EDTA and frozen and thawed for three cycles in dry ice/methanol. Lysozyme (Sigma) was then added to 200 μg/ml, the samples were incubated at 4°C or 37°C for 30 min, sonicated briefly to reduce viscosity and filter sterilized before biological assay.

Strains JM107(pLB5001-4) and JM107(pRK248cIts)(pLNompGM), which contain secretion vectors, were propagated and induced in M9NAM medium [M9 medium (9) with 0.4% glucose, 22 μg/ml methionine, 0.02X methionine assay medium (Difco), and 5 μg/ml thiamine] plus appropriate antibiotics. The strain containing (pLNompGM) was induced and harvested as described for (pLNGM). The strain containing (pLB5001-4) was prepared

for induction as described previously with the exception that all growth was at 37°C and induction was begun by the addition of 2 mM IPTG (Bethesda Research Laboratories).

All *E. coli* samples discussed were harvested under growth conditions and at times after induction that maximized production of GM-CSF for that particular strain and expression plasmid.

Generation of Rabbit Antihuman GM-CSF Antiserum

New Zealand adult, female rabbits were immunized subcutaneously with 250 μg of yeast-produced rGM-CSF protein emulsified in Freund's complete adjuvant, subcutaneously (SC) in two sites. Approximately 1 month later, the animals were boosted with 200 μg of GM-CSF emulsified in Freund's incomplete adjuvant (FIA), SC in two sites. The animals were then bled every 2 weeks and the serum tested for GM-CSF antibodies by dot blot analysis. One rabbit demonstrated a modest titer to GM-CSF beginning at week 6, following the primary immunization. This animal was subsequently injected with an additional 100 μg of GM-CSF protein in FIA at week 12. Serum titers to GM-CSF were monitored over the course of immunization and for 8 weeks after (through week 20). Titers were consistently higher than 1:2500 against GM-CSF protein as assayed by dot blot analysis.

Generation of Monoclonal Antibody to GM-CSF

Balb/c female mice (8–12 weeks of age) were immunized with 50 μg of yeast-produced rGM-CSF protein emulsified in Freund's complete adjuvant subcutaneously, in the hind footpads. Approximately 3 weeks later an animal, showing reactivity to GM-CSF protein by dot blot (detailed later), was boosted with 100 μg of GM-CSF protein intravenously. Four days after the intravenous injection with GM-CSF, the animal was exsanguinated and the spleen cells fused to the myeloma cell line NS-1. Ten to fourteen days later, culture supernatants were tested for reactivity to human GM-CSF protein by the dot blot assay. Of approximately 600 wells tested, one hybridoma supernatant, designated 3G11, was consistently reactive to GM-CSF protein. Following cloning by limiting dilution, this cell line was further characterized and ascites produced. The resulting antibody preparation was then purified from ascites as described previously by Dower et al. (27).

Immunological Detection of rGM-CSF

The amount of rGM-CSF in conditioned yeast medium and *E. coli* extracts was determined quantitatively by enzyme-linked immunoabsorbent assay (ELISA) using the dot blot procedure described by U:dal et al. (28) and Conlon et al. (29) with the following variations. One-microliter aliquots of serially diluted yeast supernatants in phosphate-buffered saline (PBS) were spotted onto a nitrocellulose filter (Schleicher and Schnell BA85) alongside serial dilutions of homogeneous preparations of purified rGM-CSF. *Escherichia coli* extracts prepared by SDS lysis [2% SDS, 62.5 mM Tris (pH 6.8), 1% β-mercaptoethanol] were serially diluted in PBS containing 2% SDS. The filter was air dried and placed in "blocking" buffer consisting of 3% bovine serum albumin (BSA) in PBS for 1 hr. The filter was then sequentially exposed (30) to antihuman GM-CSF antibody 3G11, goat antimouse IgG conjugated to horseradish peroxidase, and color-developing solution according to the manufacturer's specifications (BioRad). Western blot analysis of total cellular protein of *E. coli* extracted with SDS lysis buffer containing 10% glycerol, boiled for 5 min and separated by SDS-PAGE was performed as described earlier following electrophoretic transfer (Hoeffer) to nitrocellulose sheets by methods supplied by the manufacturer. Quantitation of rGM-CSF content in *E. coli* extracts was also performed by Western blot analysis in which serial dilutions of samples were electrophoresed alongside dilutions of purified rGM-CSF standards. A single species of 3G11 reactive material of the predicted molecular mass was evident in the *E. coli* extracts. Control samples of either yeast supernatants or *E. coli* extracts prepared from non-rGM-CSF-producing cultures did not produce a 3G11-dependent signal in either immunological procedure.

Assay of Tumoricidal Activity

Human peripheral blood monocytes were prepared from Ficoll-Hypaque-purified peripheral blood leukocytes by Percoll density gradient centrifugation (31). These cells were then allowed to adhere in 96-well culture plates for 1 hr in RPMI with 5% fetal bovine serum. Nonadherent cells were removed by three washings. The adherent population was found to be greater than 95% monocytes as judged by Wright-Giemsa stain. Adherent cells were treated for 24 hr with the indicated additive, at which time the culture medium was replaced and [^{125}I]iodeoxyuridii.e-labeled A375 target cells were added. After an additional 72 hr, residual adherent A375

cells were harvested and counted. Earlier studies (32) showed that more than 90% of the ^{125}I present in the supernatants of these cultures is in soluble form and the rest is associated with dead cells and debris. Percentage cytotoxicity was calculated as: 100 X [1-(cpm in target cells cultured with activated monocytes/cpm in target cells cultured with control monocytes)].

RESULTS AND DISCUSSION

Complementary DNA Cloning and Analysis of the GM-CSF Gene

The procedures used for isolation and analysis of the human GM-CSF gene have been described (4). A DNA fragment from the coding region of a mouse GM-CSF cDNA was used to probe a human cDNA library. Clones were isolated that contained the entire coding region and most of the non-coding regions for human GM-CSF mRNA (see Fig. 1).

Ribonucleic acid from HUT-102 cells (an HTLV-1-t-transformed T-cell lymphoma) and from T lymphocytes activated with mitogen plus concanavalin A was analyzed by Northern blot using an SP6-derived GM-CSF-specific probe. A single size of mRNA of approximately 900 nucleotides was found. Analysis of genomic DNA by Southern hybridizations indicated that the GM-CSF gene is probably a single copy gene (4).

Expression of Recombinant GM-CSF in Yeast

GM-CSF has been synthesized and secreted by the yeast *S. cerevisiae* under control of either the α-factor promoter or the alcohol dehydrogenase 2 (ADH2) promoter and the α-factor leader peptide (see Fig. 2). The α-factor leader is a peptide of 84 amino acids capable of directing the secretion of foreign proteins (13,14). The yeast expression vector shown in Fig. 2 also contains sequences necessary for selection and replication in both *E. coli* and *S. cerevisiae*.

Appropriate yeast strains (*trp⁻*) containing either (pYαHuGM) or (pαADH2HuGM) (see Fig. 2) secrete mature, biologically active GM-CSF into the culture medium in very low amounts (Table 1, columns A and C). However significantly higher levels of cross-reacting material (CRM) are detected serologically by ELISA (see Table 1, columns B and D). Little or no GM-CSF activity could be demonstrated intracellularly.

It was thought that proteolytic degradation of the GM-CSF, perhaps during the secretory process, was a major factor limiting yields. During

Table 1 Production of Recombinant GM-CSF by Yeast

| | μg Secreted GM-CSF/ml of medium | | | |
| | α-factor promoter | | ADH2 promoter | |
	A[a]	B[b]	C[a]	D[b]
GM-CSF (wild type)	0.05-0.07	4-6	0.5-1.0	20-30
GM-CSF (Leu-23)	0.5-1.0	2-3	5-13	25-35

[a] Fluorescamine assay of HPLC-purified, biologically active GM-CSF.
[b] Cross-reacting material detected by ELISA.

the secretory process, the α-factor leader is removed proteolytically by the KEX2 gene product after the dibasic residues Lys-Arg (33,34). At least one other yeast protease capable of recognizing dibasic residues has also been identified (35). The presence of a dibasic Arg-Arg, which can also serve as a KEX2 substrate (34), at amino acids 23 and 24 of mature GM-CSF suggested that this protein might be sensitive to one of the yeast proteases. To test this possibility, the arginine at position 23 was changed to a leucine by in vitro mutagenesis (21). This change makes human GM-CSF homologous to murine GM-CSF at the altered position. The loss of the dibasic residues resulted in approximately a tenfold increase in secreted, biologically active GM-CSF (see Table 1, column A).

Table 1 also compares the expression of GM-CSF under control of the constitutive α-factor promoter and the glucose-repressible ADH2 promoter (10,11). Increased expression of both the wild-type and mutant (Leu-23) GM-CSF was obtained with the ADH2 promoter by a factor of 5 to 10. This could be due to differences in the promoter strengths, the availability of positive regulatory elements (both promoters require positive activation by factors provided by single-copy chromosomal genes), or the interaction of the specific promoter with the foreign gene.

Secretion of proteins from yeast, including the glycosylation process, takes place by a mechanism similar to that found in mammalian cells (36). The predicted molecular mass of the unglycosylated, mature GM-CSF is 14,476. Analysis of secreted GM-CSF from yeast by SDS-PAGE and

Western blots revealed GM-CSF of heterogeneous molecular mass with major species at M_r 17,000 and M_r 25,000, indicative of glycosylated protein. Some hyperglycosylated material M_r 35,000-40,000 was also observed. The high-molecular-weight forms of GM-CSF have been shown to be susceptible to glycanase, an enzyme similar to endoglycosidase F that hydrolyzes both high mannose and complex asparagine-linked sugars. Glycanase treatment of secreted GM-CSF resulted in biologically active material with a molecular mass of 14,500-17,000, demonstrating N-linked glycosylation.

Amino acid sequence analysis of purified, secreted GM-CSF from yeast revealed two forms of the protein. Approximately 60% of the GM-CSF had the expected amino-terminal sequence for mature GM-CSF of Ala-Pro-Ala-Arg-Ser-Pro, while 40% was missing the amino-terminal Ala-Pro pair. Proteolytic cleavage of the Ala-Pro amino acid pair from the NH_2-terminus of this protein is consistent with the report by Achstetter et al. (37) of a class of dipeptidyl aminopeptidases in yeast. They have demonstrated both the presence of a cytoplasmic dipeptidyl aminopeptidase II, which has been shown to be active on Ala-Pro-containing artificial substrates, and an aminopeptidase P from membrane fractions that is active on the Ala-Pro artificial substrate. We are currently investigating the use of an aminopeptidase P-minus strain to alleviate NH_2-terminal proteolysis.

Expression of Recombinant GM-CSF in E. coli

Expression of GM-CSF in E. coli

Expression of GM-CSF in E. coli was initially performed by inserting the coding sequence into an expression vector that had given high-level expression of the human lymphokine IL-1β (Kronheim et al., in preparation) and of bovine IL-2 (16). The vector, designated (pLNGM), utilizes the strong leftward promoter, P_L, of bacteriophage lambda and a synthetic consensus sequence for initiation of translation followed by the GM-CSF-coding sequence, and terminating with the bacteriophage lambda transcription terminator, t11 (see Fig. 3). Induction of the P_L promoter in E. coli strain RRI(pRK248cIts) resulted in production of GM-CSF. Analysis of extracts from induced E. coli cultures showed production of approximately 1×10^4 units of GM-CSF activity (CFU-C) per milliliter of culture medium as measured in a human bone marrow colony formation assay (4). However the level of production of GM-CSF was too low to allow visualization of the protein on SDS-PAGE analysis of total E. coli proteins.

A number of additional plasmids were constructed utilizing alternative translation initiation regions, but none of these gave increased expression

of GM-CSF. Use of alternative codons for a substantial portion of the coding region also resulted in no increase in the level of production of GM-CSF. These results led to the suggestion that poor production was caused by instability of rGM-CSF protein in E. coli because of endogenous proteases. To explore this possibility the plasmid (pLNGM) was transferred into the E. coli strain CAG629 which is a lon^-, $htpR^-$ double mutant conferring reduced levels of one or more proteases (18). The strain was transformed initially with the plasmid (pACYC184clts), which encodes a bacteriophage lambda temperature-sensitive cI repressor of the P_L promoter. Transfer of (pLNGM) into this strain followed by induction by temperature shift resulted in GM-CSF activity at a tenfold higher level (approximately 1×10^5 CFU-C/ml) than with strain RRI. Total E. coli cellular protein was analyzed by SDS-PAGE and silver staining as described previously (16). Protein profiles from induced cultures harboring (pLNGM) contained a band of the predicted molecular mass for nonglycosylated GM-CSF (M_r 14,476), whereas this peptide species was absent from control cultures in which (pLNGM) was substituted with a control plasmid (pPL-λ). The level of GM-CSF was determined by quantitative Western blots using the monoclonal antibody 3G11. The induced E. coli cultures contained approximately 5-10 μg GM-CSF/ml of culture. Determination of the total protein content of these cultures showed that the GM-CSF corresponds to 0.15-0.3% of the total E. coli cellular protein.

If sensitivity to cytoplasmic proteases is a major factor limiting production of GM-CSF in E. coli, then secretion of the protein out of the cytoplasm and into the periplasmic space by use of a signal sequence should increase the level of the protein. We therefore inserted the coding sequence for GM-CSF into a secretion expression vector developed by Ghrayeb et al. (20). This vector, (pIN-III-omp-a3), allows expression of inserted genes under the control of both the E. coli lipoprotein promoter (lpp) and the lac promoter-operator. Expression is regulated by the lac repressor which is also produced by the vector. Translation initiation and secretion are controlled by sequences from the E. coli ompA gene, which codes for a major outer membrane protein. The sequence of the vector (pL.BS001-4) (see Fig. 4) upstream of the coding sequence for mature GM-CSF was altered by in vitro mutagenesis to remove four extra codons at the junction of ompA and GM-CSF sequences to allow proper proteolysis of the ompA signal peptide from mature GM-CSF (Libby et al., in preparation). Induction with IPTG of E. coli JM107 containing (pLBS001-4) and analysis of cellular protein on SDS-PAGE showed production of GM-CSF.

Immunological detection by dot blots and quantitative Western blots revealed one band of the predicted molecular mass at the level of 5-10 μg rGM-CSF/ml of culture medium which corresponded to 0.25-0.5% of the total cellular protein. This level of production of GM-CSF is similar to the level of GM-CSF produced by (pLNGM) in the protease-deficient strain but is much greater than the level of GM-CSF found after induction of (pLNGM) in strains such as RR1, containing wild-type levels of cytoplasmic proteases.

To determine whether the increased level of GM-CSF produced by (pLBS001-4) was primarily due to secretion or to the use of the lpp promoter, we constructed an additional secretion vector, (pLNompGM), which used the P_L promoter and transcription terminator as in (pLNGM) but contains the modified DNA sequence for the $ompA$ signal sequence and translation initiation region (see Fig. 3). Induction of $E.\ coli$ strain JM107 (pRK248cIts) containing (pLNompGM) and analysis as before showed expression of GM-CSF at a level of approximately 4-8 μg/ml of culture, which corresponded to 0.13-0.25% of cellular protein.

Therefore it is probable that secretion of GM-CSF out of the $E.\ coli$ cytoplasm was the primary factor increasing GM-CSF production in (pLBS001-4). We have not eliminated the possibility that differences in the translation initiation regions of the constructs affected the levels of GM-CSF.

Amino acid sequence analysis of rGM-CSF produced by (pLBS001-4) and purified to homogeneity has shown the material to be completely processed at the predicted alanine (see Fig. 4; Libby et al., in preparation).

Effect of GM-CSF on Macrophage Tumoricidal Activity

It was traditionally thought that the primary function of CSFs was to promote the growth and differentiation of hematopoietic precursor cells. It is now becoming increasingly clear that CSFs can also affect survival and activation of cells of hematopoietic origin. Previous studies have indicated that GM-CSF can induce antibody-dependent cellular cytotoxicity (38), the killing of schistosomula by neutrophils and eosinophils (39), and the intracellular killing of $Leishmania$ spp. by macrophages (40). We have recently shown that rGM-CSF can also stimulate nonspecific tumoricidal activity of monocytes in vitro (41).

Purified, rGM-CSF produced by yeast induced peripheral blood monocytes to express tumoricidal activity against the malignant melanoma cell line A375 (Table 2). Lipopolysaccharide (LPS) was not required for the function of GM-CSF in this assay, and added LPS had no stimulatory effect.

Table 2   Activation of Peripheral Blood Monocytes for Tumor Cytotoxicity in vitro by GM-CSF and IFNγ

| | % Cytotoxicity | | | | |
| | Level of LPS (ng/ml) | | | | |
Lymphokine	None	10.0	1.0	0.1	0.01
None	0	83	79	39	0
GM-CSF, 500 CFU-C/ml	36	36	36	36	0
IFNγ, 100 U/ml	8	82	15		

Purified peripheral blood monocytes were prepared and cultured as described in Materials and Methods in the presence of lipopolysaccharide, purified natural IFNγ, or purified GM-CSF as indicated. After 24 hr, the culture medium was replaced and [125I]-labeled A375 target cells were added. After an additional 72 hr, residual adherent A375 cells were harvested and counted. Units of IFNγ were determined with a virus plaque-reduction assay and comparison with an NIH international IFNγ standard.

This is in marked contrast to the requirement for suboptimal levels of LPS for stimulation by γ-interferon (IFN-γ) of monocyte killing of tumor cells (see Table 2; Ref. 42). Preparations of rGM-CSF used in these tests were shown to be free of endotoxin (less than 1 pg/μg rGM-CSF). The GM-CSF-activated monocytes were also able to lyse several other tumor targets including a murine melanoma and a human bladder carcinoma.

This regulation of monocyte activity by GM-CSF may provide a mechanism whereby T lymphocytes, in response to antigen, may regulate a nonspecific cytotoxic reaction by macrophages against tumors.

SUMMARY

Isolation of cDNAs for GM-CSF has allowed analysis of the gene and its expression in various human cell populations. Initially, low-level expression in yeast was increased by manipulation of the coding sequence and the specific promoter used. Increased expression in $E.\ coli$ was obtained by use of a protease-deficient strain or by addition of a signal sequence for secretion of the protein out of the cytoplasm. Availability of the recombinant protein permits more definitive studies of its structure and function than could be performed with naturally occurring material. An example of

such studies is our recent analysis of the stimulatory effect of the recombinant protein on monocyte killing of tumor cells. Such regulation of macrophage activation by GM-CSF may represent an important pathway of antitumor defense.

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MOLECULAR CLONING AND STRUCTURAL ANALYSIS OF NEAR FULL-LENGTH DNA COMPLEMENTARY TO THE mRNA CODING FOR BOVINE PARATHYROID HORMONE. David F. Gordon,* Christine A. Weaver,* and Byron Kemper. University of Illinois, Urbana, IL, 61801

To facilitate studies on the structure and function of the gene for parathyroid hormone we have synthesized and cloned in bacteria DNA complementary to mRNA coding for bovine parathyroid hormone (PTH). Single-stranded DNA complementary to PTH mRNA contained about 730 bases. After synthesis of the second strand with E. coli DNA polymerase and treatment with S1 nuclease to cleave the hairpin loop, double-stranded DNA of about 700 base pairs was obtained. The double-stranded cDNA was analyzed by digestion with restriction endonucleases before and after cleavage of the hairpin loop. Restriction sites for Alu I, Mbo II, Hau 3A, Sau I and Taq I enzymes were present in PTH cDNA. The sizes of the restriction fragments corresponding to the 5' terminus of the mRNA were about 135, 220 and 315 bases for the Sau 3A, Sau I and Taq I endonucleases, respectively. One Taq I site, two Sau I sites and several sites for the other enzymes were present. PTH cDNA was inserted into the Pst I site of pBR322. About 150 and 300 bacterial clones carrying recombinant plasmids were obtained by A·T and G·C tailing methods, respectively, from 15 ng of PTH cDNA. Plasmids with the largest inserts were selected by agar gel electrophoresis and those containing PTH cDNA were selected by restriction endonuclease analysis. We are presently analyzing the structures of these plasmids in detail. (Supported by an USPHS NIH Grant)

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STRUCTURAL FEATURES OF THE GOAT β-GLOBIN GENES, INCLUDING TWO PSEUDOGENES. Eric A. Schon*, Joel R. Haynes*, Paul Rosteck, Jr.*, Patricia M. Gallagher*, Michael L. Cleary*, and Jerry B. Lingrel. University of Cincinnati Med. Sch., Cincinnati, Ohio 45267.

The genes coding for the goat fetal (γ), juvenile (β^C), adult (β^A), and presumed embryonic (ε) β-globins have been isolated and identified from a goat DNA recombinant library.

In order to obtain insight into the control of this developmentally regulated set of genes, the 5' region immediately prior to the translation initiation sites of the β^C and β^A genes were sequenced. Surprisingly, the 5' regions of these two genes, which are expressed at two distinct periods in the development of the goat, contain identical sequences for 154 nucleotides. Thus, the site of regulation of the switch from juvenile to adult β-globin is not in the immediate 5' vicinity of these two genes.

In addition to the four β-globin genes noted above, at least two other β-globin-like sequences have been identified on the 5' side of the β^C gene. These two pseudogenes appear not to code for any known globin, because both contain improperly placed translation termination codons. (Supported in part by NIH Grant #AM 20119)

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CLONING AND CHARACTERIZATION OF A 6.7 KILOBASE FRAGMENT OF RAT RIBOSOMAL DNA. Lawrence I. Rothblum*, Ramachandra Reddy* and David L. Parker*. (CIOR; Kurt Randerath). Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

Digestion of rat DNA with the restriction endonuclease Eco R₁ generated three fragments which hybridized to ribosomal RNA. 11-13 kilobases, 6.7 kilobases and approximately 4.5 kilobases. To study the processes of transcription and maturation of rRNA, the molecular cloning of the rat ribosomal DNA was undertaken. Rat nucleolar DNA was digested with the restriction endonuclease Eco R₁, and cloned with the λkb vector λgt WES·λB. Approximately 300 recombinants were screened by in situ hybridization with 5'-(³²P)-end-labeled ribosomal RNA. Two positive clones were identified and further characterized. The inserted fragment of both clones (λR₂D₆ and λR₂E₁₁) was found to be the same size, 6.7 kilobases, and both clones hybridized to 18S, 28S and 5.8S ribosomal RNA. Using dual and single terminal labeled fragment(s), preliminary mapping of the inserted fragment was carried out with the restriction endonucleases Hind III(a), Bam HI(b), Bgl II(c) and Pst I(d), to yield a restriction map of the fragment:

The cloned fragment has been further characterized by correlating the restriction endonuclease map with Southern blots carried out with 18S, 28S and 5.8S rRNA probes.

Complete Amino Acid Sequence of Human Parathyroid Hormone[†]

Henry T. Keutmann,* Mary M. Sauer, Geoff N. Hendy, Jeffrey L. H. O'Riordan, and John T. Potts, Jr.

ABSTRACT: Structural analysis of human parathyroid hormone has been hindered by the scarce supplies of available starting material, tissue pooled during surgery for hyperparathyroidism. Using successive lots of hormone extracted and purified from this source, we have completed the amino acid sequence of the 84-residue molecule by means of Edman degradation of tryptic peptides and their subfragments. The technique of biosynthetic labeling with specific radioactive amino acids was extensively used to complement the analyses of extracted hormone. The human parathyroid hormone molecule differs at 11 sequence positions from the bovine and from the porcine hormones. The carboxyl terminus, while highly conserved overall, contains changes at positions 79 and 83 which may be sufficient to effect observed alterations in immunological cross-reactivity with antibovine antisera directed toward this region. The most marked sequence differences occur through the middle portion of the molecule, where six of the eight residues in the segment 40-47 differ from either or both of the other species. Proline residues are especially abundant in this region of the human hormone. These changes could confer significant conformational differences to the human molecule which would be expected to further influence immunoreactivity and perhaps also the nature of enzymatic cleavages occurring during in vivo metabolic degradation of the hormone.

The complete sequences of parathyroid hormone from two species, bovine (Brewer and Ronan, 1970; Niall et al., 1970) and porcine (Sauer et al., 1974), have been established using hormone obtained from glands available as a byproduct of meat processing, but progress in the structural analysis of the human hormone has been restricted by the need to accumulate human tissue gradually during surgery in patients with hyperparathyroidism.

Several years ago, the sequence of the biologically active amino-terminal one-third of the molecule was reported by Brewer and co-workers (1972) and by ourselves (Niall et al., 1974). The sequence of this region was determined first because of its accessibility to automated Edman degradation. There were, however, discrepancies between the two proposals involving residues 22, 28, and 30; reexamination of the amino-terminal region by the respective laboratories has so far failed to reconcile these differences (Brewer et al., 1975; Keutmann et al., 1975). Nevertheless, a variety of physiological and immunological studies have ensued using synthetic peptides comprising the active region (Tregear et al., 1974).

There has been a clear need for structural information concerning the remainder of the molecule, in light of the numerous ongoing investigations concerning structure-function relations (Rosenblatt et al., 1976), conformation (Fiskin et al., 1977), peripheral metabolism (Segre et al., 1977), and radioimmunoassay of the hormone in blood for physiological and clinical studies, since there is extensive evidence that the predominant circulating form of hormone is a carboxyl-terminal fragment [see Habener and Potts (1976) for a review].

We, therefore, prepared successive lots of purified human parathyroid hormone (hPTH)[1] as sufficient quantities of adenoma tissue were accumulated. In the course of this, we were

able to further define conditions for isolation and to obtain definitive compositional analysis of the molecule (Keutmann et al., 1978). These preparations were then employed in structural analysis of the middle and carboxyl-terminal regions, in conjunction with microsequencing techniques utilizing hormone biosynthetically labeled with radioactive amino acids.

As a result of these combined analytical approaches, the sequence of the entire 84-residue molecule has been completed. This report provides a detailed description of these structural studies as well as an assessment of how sequence differences from the animal hormones might influence the comparative physiological and immunological properties of human parathyroid hormone.

Materials and Methods

Hormone Preparations. Successive lots of human parathyroid hormone were extracted from pooled adenomatous and hyperplastic tissue by means of phenol, processed into a trichloroacetic acid precipitate, and purified by gel filtration on Bio-Gel P-100 and ion-exchange chromatography on carboxymethylcellulose. Details of these procedures have been published previously (Keutmann et al., 1974, 1978).

Biosynthetic Labeling. Human parathyroid adenoma tissue, freshly obtained from surgery, was sliced and incubated with the appropriate radioactive amino acids in Earl's balanced salt solution (Gibco) as previously described (Keutmann et al., 1975). Labeled amino acids, obtained from New England Nuclear (Ala, Pro, Gln, Lys, Leu, and Thr) or Amersham-Searle (Phe) were used in quantities of 25 μCi/mL of medium for ^{14}C and 150 μCi/mL for [3]H. The remaining, unlabeled amino acids were added to a concentration of 5 × 10^{-4} M. The tissue was extracted with 8 M urea/0.2 N HCl, combined with the medium, and precipitated with Cl₃AcOH as described by Kemper et al. (1972). The pooled Cl₃AcOH preparations from multiple incubations with a given set of amino acids were

[1] Abbreviations used: hPTH, human parathyroid hormone; Tos-PheCH₂Cl, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; Cl₃AcOH, trichloroacetic acid.

further purified by gel filtration and ion-exchange chromatography (Keutmann et al., 1975) in the presence of bovine parathyroid hormone as carrier.

Enzymatic Cleavage Procedures. Tos-PheCH₂Cl-trypsin and chymotrypsin were each obtained from Worthington, and thermolysin was obtained from Calbiochem. In each case, the digestions were carried out at an enzyme/substrate ratio of 1:100 (M/M) in 0.1 M ammonium bicarbonate buffer (pH 8.7), for 1 h at 37 °C. Staphylococcal protease (Miles Laboratories) was used at a molar ratio of 1:50 in the same buffer at 37 °C for a 22-h time period.

ε-Amino Group of Lysine Blockade. Lysine residues were blocked in order to limit tryptic cleavage to arginine residues. Parathyroid hormone was treated with a 40-fold molar excess of maleic anhydride for 20 min at pH 9.0, 20 °C (Klotz, 1967), and separated from the reagents by gel filtration on Sephadex G-25.

To reexpose lysines for further tryptic digestion, maleoyl groups were removed by treatment with 0.1 N formic acid for 45 min at 80 °C.

Edman Degradation. All degradations were done by the three-stage manual Edman procedure (Edman, 1960; Niall and Potts, 1970). Radioactive phenyl [³⁵S]isothiocyanate (Amersham-Searle) was used as a coupling reagent for increased sensitivity (Jacobs and Niall, 1975). Phenylthiohydantoins were identified by thin-layer chromatography (Edman and Begg, 1967) (followed by autoradiography) or by gas chromatography (Pisano and Bronzert, 1969). Yields of phenylthiohydantoins at successive cycles were quantitated either from the gas chromatographs or, in the case of radioactive derivatives identified by TLC, by counting after scraping from the thin-layer plate.

Edman degradations of biosynthetically labeled peptides were done under the same conditions, except for the use of unlabeled phenyl isothiocyanate. Aliquots of the ethyl acetate phase containing the radioactive phenylthiohydantoin were counted for ³H and ¹⁴C activity by scintillation counting. Whenever possible, the identification of the radioactive phenylthiohydantoin was confirmed by counting after thin-layer chromatography.

Amino Acid Analysis of Peptide Fragments. Acid hydrolysis was carried out in 5.7 N HCl at 110 °C for 24 h in vacuo, in the presence of 1:2000 (v/v) mercaptoethanol. Analyses were performed using the Beckman Model 121 MB automatic analyzer. Amino acids were normalized by best fit based upon recovery of all stable residues.

To establish the carboxyl-terminal residue surviving after Edman degradation carried to the penultimate residue of a fragment, the final reaction mixture was dissolved in analyzer buffer and applied directly to the column without hydrolysis.

Column Procedures. Column chromatography employed Sephadex (Pharmacia) or Bio-Gel (Bio-Rad) resins eluted with ammonium acetate buffer (0.14 M, pH 5.0). Ammonium bicarbonate buffer (0.1 M, pH 8.7) was used for elution of maleoylated peptides. All columns were 0.9 × 70 cm in size, run at 20 °C. Specific column chromatographic separations are described further under Results. Column elution was monitored by optical density at 206 and 280 nm by use of the LKB "Uvicord" Model 2089 continuous monitor. Biosynthetically labeled preparations were also monitored by scintillation counting of aliquots (usually 25 µL) from successive eluate tubes.

Thin-Layer Techniques. Thin-layer chromatography (TLC) was done in the solvent system butanol-water-pyridine-acetic acid (150:120:50:30) using Merck 100-µm glass-backed cel-
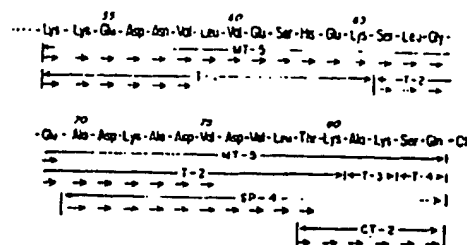
FIGURE 1: Sequence analysis of the 53-84 fragment (peptide MT-5) of hPTH. Peptide subfragments employed in the analysis are designated beneath the sequence by the following code: MT, maleoylated tryptic; T, tryptic (after removal of maleoyl groups from lysines); SP, staphylococcal protease; CT, chymotryptic. Arrows denote residues identified by manual Edman degradation.

lulose plates, with 20-30 µg of sample applied to the plates for analytical runs. For preparative scale chromatography, 100 µg of sample was striped along 1.5 cm of the origin, with guide spots at either side for identification.

Thin-layer electrophoresis (TLE) was done using the same plates in a buffer system butanol-pyridine-water (1:30:270), pH 6.5. Plates were run for 40 min at 4 °C, 600 V, 11 mA, using the Camag electrode system. All plates were stained with ninhydrin.

Synthesis of Fragment 38-44. The fragment (38-44) used in enzymatic cleavage studies was prepared by the solid-phase technique (Merrifield, 1967) as described in detail by Tregear et al. (1974). The peptide was purified by gel filtration on Bio-Gel P-2 in 1 N acetic acid and adsorption chromatography on a column of Merck silica gel 60 using the same buffer system as that employed for TLC (above).

Results

In undertaking the structural analysis, knowledge of the tactics useful in sequence analysis of the homologous bovine and porcine hormones suggested that the human hormone was most readily analyzed by separate regions: the amino terminus, previously completed by automated Edman degradation of the intact hormone; the carboxyl terminus, comprising residues 53-84; and the middle region, commencing in the vicinity of residue 35 (end point of the earlier amino-terminal analyses) and extending through residue 52. The carboxyl-terminal and middle regions coincided with the pattern of cleavage obtained from tryptic cleavage of maleic-blocked hormone. This had been shown earlier (Keutmann et al., 1975, 1978) to take place at arginine residues positioned identically to the bovine and porcine hormones.

Multiple peptide subfragments were isolated, analyzed for composition, and degraded by the Edman technique as extensively as possible to provide sequence overlap. Biosynthetic labeling was used to permit high-sensitivity microsequencing of peptide fragments, especially in difficult regions of sequence analysis. Mixture degradations—analyses of several peptides simultaneously without separation of component fragments (Gray, 1968)—were done as a confirmation of most sequence positions. The combined results are presented first for the carboxyl and then the middle region of the molecule.

The Carboxyl Terminus (53-84). Residues 53-69. The overall strategy for analysis of the carboxyl-terminal region is outlined in Figure 1. It was based on the fragment 53-84 prepared by tryptic digestion of maleoylated hPTH and separated by gel filtration on a column of Sephadex G-50 (Figure 2). The fragment prepared from 1.5 mg (150 nmol) of hPTH was degraded for 17 cycles, through residue 69, by the manual Edman procedure. Location of histidine at position 63 had been
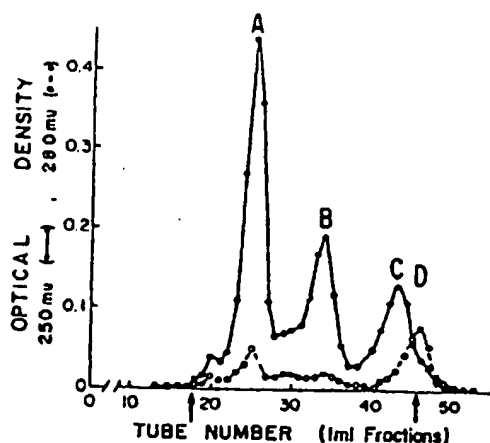
FIGURE 2: Sephadex G-50 gel filtration of a tryptic digest of human parathyroid hormone after maleic blockade of lysine residues. Cleavage of arginine residues at positions 20, 25, 44, and 52 produced fragments 53-84 (A), 1-20 and 26-44 (coeluting as B), 45-52 (C), and 21-25 (D). Fraction size was 1-mL. Arrows denote void and salt volumes of column, respectively.
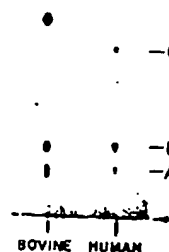

FIGURE 3: Thin-layer chromatography of tryptic subdigests of fragment 53-84 (MT-5) from bovine and human parathyroid hormone. Component A represented fragment 53-65 and component C fragment 66-80. Component B proved to represent the two dipeptides 81-82 and 83-84 (see text).

separately identified in other studies (Segre et al., 1977) using automated degradation of the peptide isolated after labeling with radioiodine.

Residues 70-75. The sequence was continued toward the carboxyl terminus by use of peptides prepared by tryptic subdigestion of another preparation of the 53-84 fragment, after reexposure of lysine residues by treatment with 0.1 N formic acid. Thin-layer chromatography of the tryptic digest (Figure 3) showed three components. After elution from a preparative-scale plate, the following compositional analyses were obtained: component A: Asp₂, Ser₁, Glu₃, Val₂, Leu₁, His₁, Lys₃; component B: Ser, Glu, Ala, Lys; component C: Asp₃, Thr₁, Ser₁, Glu₁, Gly₁, Ala₂, Val₂, Leu₂, Lys₂. When the preparation was subjected to electrophoresis following chromatography, component B resolved into two peptides: Ala, Lys and Ser, Glu. This indicated a total of four tryptic peptides from the human 53-84 fragment, compared with three from the bovine. Based upon the compositions and the results of the initial Edman degradation (above), these were identified as residues 53-65 (T-1, Figure 1), 66-80 (T-2), 81-82 (T-3), and 83-84 (T-4). The tryptic fragments were separated by gel filtration on Sephadex G-25, and T-1 (53-65) was subjected to six cycles of degradation (Figure 1) in order to confirm the sequence of residues 53-58 established by degradation of the intact 53-84 peptide. Peptide T-2 (66-80) was degraded for ten cycles, extending the sequence through residue 75 (Figure 1).

Residues 79-84. Analysis of the extreme carboxyl terminus was done next, using products of chymotryptic digestion of 50 nmol of maleoylated 53-84 peptide. Only a single cleavage, between residues 78 and 79, was obtained under the relatively mild digestion conditions employed. The carboxyl-terminal hexapeptide 79-84 was separated from the longer fragment 53-78 by Sephadex G-25 gel filtration and found by composition to contain two residues of lysine and one each of threonine, serine, alanine, and glutamic acid (or glutamine). The sequence Thr-Lys-Ala-Lys-Ser- was established in five cycles of Edman degradation, and the final glutamine residue was identified directly by amino acid analysis. This sequence was found again in a second degradation of the unfractionated chymotryptic peptide mixture from digestion of another preparation of 53-84 peptide.

Since identification of the phenylthiohydantoin of maleoylated lysine is difficult to achieve unequivocally by thin-layer chromatography, the assignment of lysines was confirmed by degradation of the chymotryptic fragment (79-84) from a human hormone preparation biosynthetically labeled with [¹⁴C]lysine. Counts from labeled lysines were seen at cycles 2 and 4, consistent with location of this residue at positions 80 and 82.

In the bovine hormone, the presence of proline at position 83 limited tryptic cleavage to the first of these two lysines (position 80), yielding the tetrapeptide Ala-Lys-Pro-Gln. The substitution in the human hormone of serine for proline at position 83 rendered both lysines susceptible to cleavage, producing the two fragments Ala-Lys (positions 81 and 82) and Ser-Gln (positions 83 and 84), consistent with the sequence assigned by degradation of the carboxyl-terminal chymotryptic fragment.

The glutamine at position 84 was the only glutamine residue identified during Edman degradation of the entire 53-84 region. Residue 64, glutamine in both the porcine and bovine hormone, was identified as glutamic acid in the human molecule. To confirm this finding, the biosynthetic labeling technique was used to rule out the unlikely possibility of selective deamidation. A preparation of human hormone labeled with [¹⁴C]glutamine was treated as described above to produce the 53-84 peptide, which was subdigested with trypsin after removal of maleoyl groups. The peptide subfragments were then fractionated by thin-layer chromatography and electrophoresis. Glutamine counts were associated only with the 83-84 fragment derived by electrophoretic separation of component B (Figure 3), independently confirming the absence of glutamine from position 64 as well as sites other than residue 84 within the carboxyl-terminal region.

Residues 76-78. To complete the remaining portion of the 53-84 region, staphylococcal protease was used to cleave the glutamic acid residue at position 69, as done previously in structural analysis of porcine parathyroid hormone (Sauer et al., 1974). Edman analysis of an aliquot of the product from digestion of 40 nmol of the 53-84 fragment showed cleavages at residues 55 and 61, as well as 69. The carboxyl-terminal fragment 70-84 (SP-4, Figure 1) was separated by Sephadex G-25 gel filtration and subjected to ten cycles of manual degradation, through the threonine residue at position 79. This confirmed the earlier results from degradation of tryptic fragment T-2 and provided the sequence of positions 76-78.

The Middle Region (Residues 38-52). Residues 45-52

····Asp-Val-His-Asn-Phe-Val-Ala-Leu-Gly-Ala-Pro-Leu-Ala-Pro-Arg-
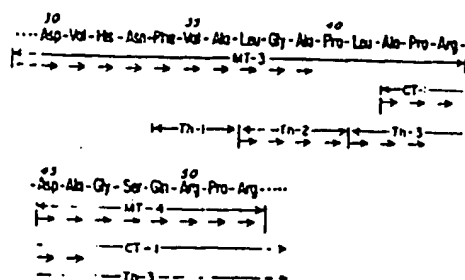

-Asp-Ala-Gly-Ser-Gln-Arg-Pro-Arg·····

FIGURE 4: Structural analysis of the middle region of hPTH. Peptides MT-3 (which originates at residue 26) and MT-4 are obtained from tryptic digestion of maleoylated hormone (see Figure 2). Other fragments isolated and used in analysis include chymotryptic (CT) and thermolytic (Th). Arrows denote residues identified by Edman degradation of the purified peptide.

FIGURE 5: Identification of residues 42 and 43 by Edman degradation of chymotryptic fragment 42-78 from human hormone biosynthetically labeled with [3H]alanine and [14C]proline. Alanine counts were found at cycles 1 (position 42) and 5 (known to be the alanine at position 46). Proline radioactivity was found at cycle 2, corresponding to position 43. This position is occupied by tyrosine in the bovine hormone.

were determined by Edman degradation of fragment MT-4 from tryptic cleavage of maleoylated hPTH. This peptide (150 nmol) was obtained from the same digest that produced the initial 53-84 preparation described above (Figure 2). The pooled 45-52 product from Sephadex G-50 was rechromatographed on Sephadex G-25 to eliminate any small amounts of fragment 21-25 remaining after the Sephadex G-50 step. The following amino acid composition was obtained: Asp, Ser, Glu, Pro, Gly, Ala, Arg₃. Seven cycles provided the sequence Asp-Ala-Gly-Ser-Gln-Arg-Pro-, and the terminal arginine was identified after direct application to the amino acid analyzer.

Residues 38-39. Sequence analysis of the amino terminus, using automated degradation of intact and cyanogen bromide cleaved hPTH, had extended from residues 1 through 37 (Niall et al., 1974). Initial efforts to extend the sequence employed fragment 26-44 from a tryptic digest of maleoylated hormone. This fragment (MT-3) coeluted from Sephadex G-50 with fragment 1-20 (Figure 2). Since the terminal α-amino group of the hormone molecule is blocked along with the ε-amino groups after maleoylation, the 1-20 fragment does not react in the Edman degradation, permitting analysis of the 26-44 fragment without separation of the two peptides. This fragment was initially employed in studies (Keutmann et al., 1975) reconfirming the sequence of residues 28 and 30. Extended degradation of a preparation of 26-44 fragment reached residue 39 (Figure 4), but extractive losses due to its markedly hydrophobic nature prevented sequence determination from progressing further.

Residues 40-44. To approach the structure of the remaining residues 40-44, products of chymotryptic and thermolytic digestion of native hormone were used (Figure 4), taking advantage of the abundance of susceptible neutral residues in this region of the molecule. At this nearly complete stage of sequence analysis, two residues of proline and one each of leucine and alanine from the compositional analysis remained to be assigned.

Pilot scale digestions of native hPTH had indicated the presence of a chymotrypsin-sensitive residue at position 41. An aliquot of 40 nmol of native hPTH was digested, and the long fragment CT-1 (residues 42-78) was separated by gel filtration on Sephadex G-50. By Edman degradation, alanine and proline were found at cycles 1 and 2 (residues 42 and 43). Arginine at cycle 3 confirmed the presence of this residue at position 44, as concluded earlier from compositional studies and the cleavage at this position by trypsin after maleic blocka-le. Aspartic acid and alanine at cycles 4 and 5 (positions 45 and 46) corresponded to those previously found in degradation of the 45-52 fragment.

Thermolysin, which cleaves the peptide bond amino terminal to neutral and aromatic residues, was next used to prepare a fragment (Th-3, Figure 4) similar to the chymotryptic peptide just described, with the addition of residue 41 at the amino terminus. This peptide extended through residue 58 and was separated best by gel filtration on Bio-Gel P-30. Leucine was found at cycle 1 of Edman degradation, followed by alanine and proline as assigned (above) to positions 42 and 43.

These results left proline as the most probable residue for the final position 40. Knowing that the already established sequence for residues 37-39 indicated exact homology between the bovine and human hormones, advantage was taken of the cleavage pattern of thermolysin to identify the peptide corresponding to this region in the bovine molecule. In preliminary studies with the bovine hormone, fragment 37-40 (Leu-Gly-Ala-Ser) was isolated from thermolysin digests of the intact molecule, eluting from Bio-Gel P-30 at a K_d of 0.8 and from subsequent TLC at an R_f of 0.62. When a comparable P-30 eluate fraction of thermolysin-cleaved human hormone was screened by TLC, a tetrapeptide with the composition (Gly, Ala, Leu, Pro) was located with a similar chromatographic mobility (R_f 0.64). This fragment (Th-2, Figure 4) was isolated by preparative-scale TLC. Three cycles of Edman degradation yielded the sequence Leu-Gly-Ala- and the final cycle, corresponding to residue 40, was identified as proline by amino acid analysis.

It was possible to rely extensively on biosynthetic labeling for separate verification of the sequence Pro-Leu-Ala-Pro thus found for residues 40-43. Residue 41 was confirmed by thermolysin digestion of a [3H]leucine-labeled human hormone preparation. Leucine radioactivity eluted with the fragment 41-58 (Th-3), and these counts were localized to cycle 1 by Edman degradation.

The assignment of residues 42 and 43 was confirmed by means of the biosynthetic study illustrated in Figure 5. A preparation of hPTH internally labeled with [14C]proline and [3H]alanine was digested with chymotrypsin. The 42-78 fragment (CT-1, Figure 4) was isolated as before and subjected to six cycles of Edman degradation. Alanine and proline counts were found at cycles 1 and 2, respectively, representing positions 42 and 43. At cycle 5, a second rise in tritium counts marked the alanine known to be at position 46.

In addition, it was deemed essential to eliminate directly and independently the presence of phenylalanine and threonine from this sequence region, since the content of these two amino acids found during compositional analysis of the entire mole-

cule (Keutmann et al., 1978) was somewhat in excess of the single residue of each already located elsewhere. Thus, two biosynthetic labeling experiments were carried out using combinations of these residues.

An hPTH preparation labeled with [^{14}C]phenylalanine and [^{3}H]alanine was digested with thermolysin and subjected to Bio-Gel P-30 gel filtration (Figure 6). Phenylalanine counts were found to elute exclusively with fragment 34-36 (Th-1, Figure 4) with no counts in the 41-58 fragment. Phenylalanine was thus eliminated from all positions except 34.

The presence of a threonine residue in the middle region was ruled out definitively by tryptic cleavage of maleic-blocked [^{14}C]threonine and [^{3}H]alanine-labeled hPTH. After Sephadex G-50 gel filtration, no threonine counts were found in the 26-44 region of the column, as marked by the alanine counts; all ^{14}C radioactivity eluted with the 53-84 fragment containing threonine at position 79.

The ease with which residue 41 (leucine) was cleaved by both chymotrypsin and thermolysin was surprising to us, in view of the two nearby prolines at residues 40 and 43 which might have been expected to inhibit cleavage. This prompted us to confirm this finding by studies using a synthetic peptide comprising residues 38-44 (Gly-Ala-Pro-Leu-Ala-Pro-Arg). Products of cleavage, under conditions of enzyme treatment similar to those used earlier, were assessed by TLC, amino acid analysis, and Edman degradation. Both enzymes were found to cleave at residue 41 in a manner identical to that observed in our study of the native hormone.

Discussion

The difficulties in isolating sufficient human parathyroid hormone for structural studies, which have prevented completion of the sequence analysis until now, stem from several factors. Not only is the source of adenoma tissue for extraction extremely limited, but the yield of hormone is low—approximately 0.5-1 mg/100 g of tumor tissue. Large quantities of immunoreactive hormone are lost at various steps in the extraction procedure. At least part of this represents fragments, some of which can be recovered for immunological studies (Keutmann et al., 1978; diBella et al., 1978), but it would appear that extensive fragmentation of the hormone may occur in the tissue prior to extraction. In some preparations, heterogeneity has been observed at the final ion-exchange purification step (Keutmann et al., 1978); this further reduces the yield, as separate evaluation of the different fractions is necessary. There is no evidence, however, that these fractions represent separated isohormones analogous to those demonstrated in the bovine species (Keutmann et al., 1971).

The strategy employed in our structural analysis of the overall molecule was markedly influenced by the scarcity of hormone supply. Efficient use of the extracted hormone was afforded by a high-sensitivity methodology, especially the use of radioactive phenyl isothiocyanate as coupling reagent in the Edman degradation. The conjoint use of biosynthetic labeling as a sequencing technique became particularly important as structural analysis progressed: (a) the later regions to be analyzed (such as the 40-43 segment), being the most difficult, placed extra demands on supplies of extracted hormone; (b) in later stages, the options for residues likely to occupy a given position were reduced, permitting more directed choices of labeled amino acids for incorporation; (c) the biosynthetic method provided an accurate alternative means to confirm certain residues that had proven difficult to identify by conventional methods, and to eliminate possible ambiguities due to selective deamidation.

The last named of these was especially relevant to the
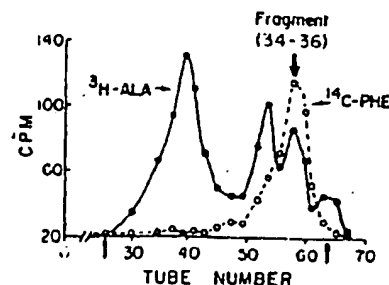
FIGURE 6: Use of a thermolysin digest of [^{14}C]phenylalanine and [^{3}H]-alanine-labeled hPTH to localize phenylalanine. When passed over Bio-Gel P-30, all phenylalanine counts eluted with fragment 34-36 (Th-1), known to contain Phe at position 34. This and other peptides were marked by alanine counts (closed circles). Arrows denote void and salt volume of column, respectively.

identification of glutamic acid at position 64, which could have arisen through loss of the side-chain amide of a glutamine initially present at this position. This possibility was examined by biosynthetic experiments employing [^{14}C]glutamine which avoid the potential problem of deamidation (Keutmann et al., 1975); the results showed glutamine to be absent from tryptic fragment 53-65. Despite this further evidence favoring glutamic acid at position 64, we plan to undertake studies using products of cell-free translation of human parathyroid mRNA (which permit equivalent incorporation of Asn, Asp, Glu, and Gln) with the aim of checking all assignments of side-chain amidated residues.

Limited tryptic digestion of maleic-blocked human parathyroid hormone was initially used in our reexamination of portions of the amino-terminal sequence (Keutmann et al., 1975, 1978) and has proven particularly appropriate in providing useful fragments in high yield for the remainder of the structure.

The entire 45-52 region and an extensive segment of the carboxyl terminus could be sequenced by degradation of these tryptic peptides. The carboxyl-terminal 53-84 fragment was completed using subdigestions with trypsin, chymotrypsin, and staphylococcal protease. The hydrophobic nature of the middle portion of the molecule called for alternative cleavage procedures. After chymotrypsin and thermolysin proved promising in pilot studies with intact bovine hormone, they were employed successfully with both extracted and biosynthetically labeled human preparations for analysis of the final 40-43 segment. Our recently reported composition of the human hormone based on multiple hydrolysates of the purified peptide (Keutmann et al., 1978) corresponds appropriately to the amino acid composition of hPTH calculated from the sequence presented here. The molecular weight of the human hormone as computed from the structure is 9427.

In the course of these studies, we have had further opportunity to examine the still disputed residues at positions 22, 28, and 30 in the amino-terminal region and have repeatedly confirmed our original findings as reported by Niall et al. (1974). Despite the obvious confusion persisting due to the unresolved differences between our results and the report of Brewer and associates (1972, 1975) concerning the nature of these three residues, we conclude from our earlier studies (Niall et al., 1974; Keutmann et al., 1975, 1978) and present findings that the structure presented here for the human hormone (Figure 7) is the correct sequence of the 84 amino acids comprising the principal, if not sole, form of parathyroid hormone obtainable from adenomatous or hyperplastic human tissue.

Figure 7 compares the sequences of the human, bovine, and
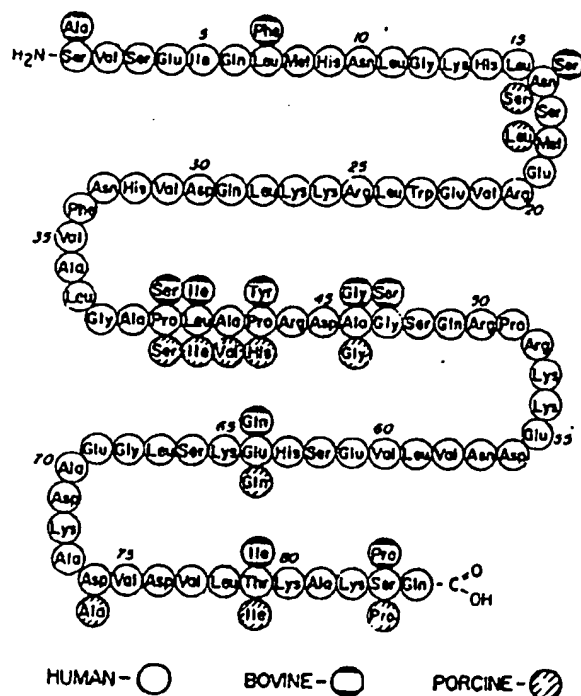
HUMAN - ○   BOVINE - ○   PORCINE - ⊘

FIGURE 7: Comparison of the amino acid sequences of human, bovine, and porcine parathyroid hormone. The human hormone is shown by the backbone sequence; substitutions found in the bovine and porcine hormones are indicated by circles alongside.

porcine molecules. The human hormone differs from each of the other species at 11 positions. Eight of these represent residues unique to the human molecule. All substitutions represent single-step mutations in the triplet-base codon, except for the substitution of proline (human) for tyrosine (bovine) at position 43. This position is unique in containing a different amino acid for each of the three species; the tyrosine found here in the bovine is the only occurrence of this residue thus far known in a parathyroid hormone molecule. Nonetheless, the human hormone, like the porcine, has proven to be a satisfactory tracer after labeling with radioiodine, which must occur predominantly at the histidine residues.

The most abundant substitutions among the three hormones occur in the midportion of the molecule: through the region 40-47, six of the eight residues in hPTH differ from either or both of the other species. Especially noteworthy is the high content of proline in one relatively short segment of the human sequence. Three residues of proline out of the 12 between positions 40 and 51 could impart marked conformational differences to this part of the molecule. This may, in turn, influence the immunological cross-reactivity between human and other species of hormone, especially with the numerous antisera currently in use that appear to be directed toward the middle region. The rate or extent of enzymatic cleavages during peripheral metabolic breakdown of the hormone, studied earlier with the bovine hormone (Segre et al., 1977), might also be affected. Although the sequences found in the human hormone at the principal sites of cleavage identified in the bovine (residues 33-34 and, secondarily, 36-37) are identical in the two molecules, the extensive substitutions in the human commencing at nearby residue 40 could influence the ease with which such cleavages take place.

Although the carboxyl-terminal region is generally well conserved, the changes that are seen at positions 79 and 83 may be sufficient to impair immunological cross-reactivity, as observed with antibovine antisera directed toward this region of the molecule (Hendy et al., 1974; Murray et al., 1975). Replacement of proline at position 83, for example, may favor extension of a region of α-helical structure (Fiskin et al., 1977) further toward the carboxyl terminus.

Our analyses show the location of charged residues throughout the human hormone molecule to be identical with the bovine, with the exception of the substitution of glutamic acid for glutamine at position 64. This difference of a single negative charge may be reflected in the finding that hPTH elutes slightly earlier than bPTH from a carboxymethylcellulose ion-exchange column (Keutmann et al., 1975). There is an additional negative charge difference between human and porcine hormone; aspartic acid, present at position 74 in hPTH and bPTH, is replaced by alanine in pPTH.

In addition to fragments and analogues based on the active amino-terminal region for use in a wide range of structure-function studies, synthetic peptides from other regions of the molecule have recently been prepared as the sequence studies have progressed, among them the 44-68 and 53-84 sequences (Rosenblatt et al., 1977, 1978) along with a number of shorter fragments. The use of one of these in supporting the current sequence analysis is described under Results. These peptides are now being widely applied to the development and characterization of antisera, in further immunological comparison between discrete regions of hPTH and bPTH, and for studies designed to discern any potential biological effects not previously recognized outside the amino-terminal region. The complete sequence of human parathyroid hormone should now permit the synthesis of additional peptides, containing the middle portion with its distinct differences from other species, for continued definition of the physiological and immunological properties of the human hormone.

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Thyroxine-Induced Conformational Changes in Prealbumin[†]

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ABSTRACT: The effects of thyroxine binding on the conformation of human prealbumin and bovine serum albumin have been examined. A blue shift in protein absorption was observed with prealbumin, whereas a red shift was observed with bovine serum albumin. In the case of prealbumin, where the two binding sites are identical, the total absorption change was confined to the binding of the first ligand and has been interpreted as resulting from a conformational change. A blue shift observed in the absorption spectrum of thyroxine, however, was the same for the first and second bound molecules. These data have been interpreted in terms of two identical and interacting sites on prealbumin and explain the origin of the difference in binding affinities between the first and second sites. Fluorescence quenching by thyroxine and thyroxine effects on tryptic hydrolysis of prealbumin are in accord with the above interpretation.

P realbumin binds 2 mol of thyroxine (T_4)[1] or T_3 with binding constants that are two orders of magnitude different for each mole (Ferguson et al., 1975; Cheng et al., 1977). The negative cooperativity observed in the binding of the two hormone molecules is not due to heterogeneity in the binding sites, since these are identical as indicated from the X-ray analysis of Blake et al. (1971, 1974). Moreover, the closest approach of the two sites is almost 10 Å (Blake and Oatley,

1977), so that a steric mechanism cannot explain the interaction between the two sites. There are binding data at several pH values with the analogue DIPA, which suggests that an electrostatic interaction may contribute to the negative cooperativity (Cheng et al., 1977). A common mechanism of developing cooperativity in subunit proteins involves a conformational change on binding a ligand on one subunit, which then alters the interactions between subunits and, consequently, the binding affinity for subsequent ligands. We have examined the binding of T_4 and DIPA to PA in order to determine whether conformational changes are produced. For comparison, the binding of thyroxine to another thyroid hormone transport protein, i.e., bovine serum albumin, was also evaluated.

Materials and Methods

Human serum prealbumin (PA), obtained from Behring Diagnostics, was purified further by gel electrophoresis, as

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(54) Title: PRODUCTION OF MATURE PROTEINS IN TRANSFORMED YEAST

(57) Abstract

A method for producing a mature protein in yeast transformed to express a corresponding precursor, wherein the mature protein sequence is contained in the precursor and is flanked proximally or both proximally and distally by a pair or triplet of basic amino acid residues. The method comprises proteolytic processing by an endopeptidase and exopeptidase present in the yeast. Yeast transformed by a plasmid containing a cDNA sequence encoding bovine preproparathyroid hormone is also disclosed.

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PRODUCTION OF MATURE PROTEINS
IN TRANSFORMED YEAST

BACKGROUND OF THE INVENTION

1. Field of the Invention.

5   This invention relates to a method for
producing a mature protein in transformed yeast
and further relates to Saccharomyces cerevisiae
transformed by a plasmid containing a
preproparathyroid hormone cDNA insert.

10   2. Description of the Prior Art.

Recombinant DNA technology now makes it
possible to isolate specific genes or portions
thereof from higher organisms, such as man and
other animals, and to transfer the genes or fragments

15   to a microorganism species, such as E. coli or
yeast. The transferred gene is replicated and
propagated as the transformed microorganism may
become endowed with the capacity to make whatever
protein the gene or fragment encodes, whether it

20   be an enzyme, a hormone, an antigen or an antibody,
or a portion thereof. The microorganism passes
on this capability to its progeny, so that in
effect, the transfer results in a new strain,
having the described capability.

25   Recombinant DNA conventionally utilizes
transfer vectors. A transfer vector is a DNA
molecule which contains genetic information
which insures its own replication when transferred
to a host microorganism strain. Plasmids are an

30   example of a transfer vector commonly used in
genetics. Although plasmids have been used as the

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transfer vectors for the work described herein,
it will be understood that other types of transfer
vectors may be employed. Plasmid is the term
applied to any autonomously replicating DNA unit
5   which might be found in a microbial cell, other
than the genome of the host cell itself. A
plasmid is not usually genetically linked to the
chromosome of the host cell. Plasmid DNA exists
as doublestranded ring structures generally on
10   the order of a few million daltons molecular
weight, although some are greater than 10^8 daltons
in molecular weight. They usually represent only
a small percent of the total DNA of the cell.
Transfer vector DNA is usually separable from host
15   cell DNA by virtue of the great difference in size
between them. Transfer vectors carry genetic
information enabling them to replicate within
the host cell.

      Plasmid DNA exists as a closed ring.
20   However, by appropriate techniques, the ring may
be opened, a fragment of heterologous DNA inserted,
and the ring reclosed, forming an enlarged molecule
containing the inserted DNA segment.

      Transfer is accomplished by a process known
25   as transformation. During transformation, host
cells mixed with plasmid DNA incorporate entire
plasmid molecules into the cells. Once a
cell has incorporated a plasmid, the latter is
replicated within the cell and the plasmid replicas
30   are distributed to the progeny cells when the cell
divides.

      Genetic information contained in the
nucleotide sequence of the plasmid DNA, including
heterologous DNA inserted into the plasmid, can
35   in principle be expressed in the host cell. The
inserted heterologous DNA typically representing

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a single gene, is expressed when the protein
product coded by the gene is synthesized by the
organism.

Once a gene has been isolated, purified
and inserted into a plasmid or other vector, the
5   availability of the gene in substantial quantity
is assured. After transfer of the vector into a
suitable microorganism, the gene replicates as
the microorganism proliferates. The vector
containing the gene is easily purified from
10   cultures of the host microorganism by known tech-
niques and separable from the vector by restriction
endonuclease cleavage followed by gel electrophoresis.
The protein product expressed by the heterologous
gene can also be recovered in substantial quantities
15   from cultures of the host microorganism by harvesting
the culture and retrieving the protein product
contained in the harvested cells. (For futher
detail of recombinant DNA technology, and an explicit
exposition of the utility of producing proteins
20   such as hormones, etc., by recombinant DNA technology,
see U.S. Patent No. 4,237,224, issued December 2, 1980
to Cohen et al., and U.S. Patent No. 4,322,499,
issued March 30, 1982 to Baxter et al. Patents
and articles cited herein are incorporated by
25   reference wherever such citations occur and shall
be considered incorporated in their entirety as if
set forth in full)..

Recombinant DNA thus holds great promise
for economically producing substantial quantities
30   of useful proteins that are difficult or costly to
isolate in such quantitites from mammalian tissue.
A major and nearly universal problem in producing
useful proteins, however, is the construction of
the actual genetic material to be inserted into the
35   transfer vector.

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      Conventional means provide for enzymatically
preparing desired genetic material by reverse
transcription. Mature messenger RNA (mRNA), which
is chemically similar to DNA and retains most of
5   the information coded in DNA, can be extracted
from tissue in which the desired gene is active.
mRNA is separated from other RNA material in the
tissue and complementary DNA (cDNA) is produced
by the enzyme reverse transcriptase, and at times
10   polymerase 1 for the synthesis of the second strand.
This cDNA, a complementary copy of mRNA and ·
similarly containing the information coded in
RNA, is often further altered in known ways to
be suitable for insertion into a plasmid vector.
15   (See W. Mahoney & S. Henikoff, Univ. of Washington
Medicine, Vol. 8, No. 4, pp. 6-14 (Winter, 1981)).
      cDNA enzymatically prepared by reverse
transcription has the potential to express a
protein chain identical to the protein expressed
20   by tissue from which the mRNA was extracted. This
alone is not sufficient, however, for the expression
of desired mature animal proteins because many
animal proteins, represented by such diverse
classes as hormones, binding proteins, enzymes,
25   antibodies, and collagen, are produced in nature
in the form of larger precursors that are
subsequently modified by cleavage to smaller
bioactive forms commonly designated mature
proteins. Thus, expression of cDNA synthesized
30   by reverse transcription only has the potential
to express the precursor of the mature protein
product.
      It has been known for several years that
bacteria such as E. coli can remove the "pre"
35   portion of its own secreted proteins. Examples
include the processing of pre-ribose binding

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protein, pre-galactose binding protein and
pre-arabinose binding protein. (L. Randall, et al.,
Eur. J. Biochem., Vol. 92, pp. 411-415 (1978);
L. Randall, S. Hardy, and L. Josefsson, Proc. Natl.
5   Acad. Sci. USA, Vol. 75, pp. 1209-1212 (1978)).
        S. Chan, et al., Proc. Natl. Acad. Sci. USA,
Vol. 78, pp. 5401-5405 (1981) has exploited the
ability of E. coli to remove the "pre" sequence.
Chan, et al., modified cDNA for human preproinsulin
10   to encode a hybrid "pre" sequence containing
portions of E. coli and mammalian "pre" sequence.
E. coli expressed the hydrid protein and correctly
removed the "pre" sequence by intra-cellular
processing. Thus, Chan, et al., was able to modify
15   human preproinsulin cDNA in a way that would allow
E. coli to produce proinsulin.
        It is also known that yeast shares the
ability to remove "pre" sequences from its own
pre-proteins. Furthermore, when an E. coli
20   preprotein was genetically engineered into yeast,
pre-B-lactamase was processed to B-lactamase.
(Roggenkamp, et al., Proc. Natl. Acad. Sci.
USA, Vol. 78, No. 7, pp. 4466-4470 (1981)).
        The above type of processing of preproteins,
25   however, will not process to mature proteins
many of the mammalian hormone precursors and
many of the other interesting mammalian protein
precursors in E. coli. These latter hormone and
protein precursors contain a "pro" portion which
30   is not processed by the enzymatic mechanism
responsible for processing the "pre" portion of
preproteins. As shown above, for example, the
natural precursor for insulin, i.e. preproinsulin
is processed in E. coli to form proinsulin.
35   Many investigators have been unable to
express pre-proteins in yeast or E. coli, let

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alone get processing. Expensive and time consuming,
investigative efforts have focused almost
exclusively on genetically eliminating the
"pre" sequences and the "pro" sequences in
5   attempting to express mature proteins without
intermediates.

In several prior art approaches, the need
for processing precursor proteins has been over-
come. Insulin is the result of natural processing
10   in human tissue involving cleaving two peptide
chains, A and B, from the single large precursor
preproinsulin and assembling the A and B chains
to form the mature hormone insulin. The A and
B chains are located within proinsulin and hence
15   E. coli which processes preproinsulin to proinsulin
does not produce the mature hormone insulin. An
approach to obtaining mature insulin using
E. coli employs chemically synthesized genes
compatible with E. coli.
20   A double-stranded synthetic DNA-coding
sequence for the insulin A chain was synthesized
chemically from fundamental nucleotide units to
yield the correct coding sequence. An extra
amino acid (methionine) was added at one end.
25   This end was fused to the bacterial gene for the
enzyme B-galactosidase which results in accumulations
of fused B-galactosidase-insulin-A-chain protein.
This same procedure was repeated for the B-chain
which resulted in the production of fused B-galactosi-
30   dase-insulin-B-chain protein.

The fused proteins are insoluble in water
and readily isolated from broken cells. The A and
B chains of insulin are released from B-galactosidase
at the extra methionine by cyanogen bromide cleavage
35   and subsequently mixed together under conditions
that allow formation of disulfide bonds between A

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and B chains, yielding mature insulin. (W. Miller,
J. of Pediatrics, Vol. 99, pp. 1-15 (1981);
D. Goeddel, et al., Proc. Natl. Acad. Sci. USA, Vol 76,
pp. 106-110 (1979)).

5   The above prior art approach overcomes the
need for processing a precursor protein, but in
turn requires processing of the fused B-
galactosidase-insulin-A-chain and B-galactosidase-
insulin-B-chain proteins to mature insulin.
10 Moreover, chemical synthesis of the DNA coding
sequences for A-chain and B-chain involves
substantial costs, even when considering that
the B-galactosidase-insulin-A-chain gene and
B-galactosidase-insulin-B-chain gene after being
15 synthesized are easily replicated for subsequent
production of insulin. (D. Williams, et al.,
Science, Vol. 215, pp. 687-689 (Feb. 1982);
W. Mahoney, Univ. of Wash. Medicine, supra).
        The approach of chemically synthesizing DNA
20 encoding for mature proteins has also been shown to
be effective for bacterial production of human
somatostation. (K. Itakura, et al., Science,
Vol. 198, pp. 1056-1063 (1977)). However, insulin
chains A and B and human somatostation are
25 relatively small sequences and chemically
synthesized DNA coded for them are relatively
small. In the case of larger proteins, chemical
synthesis of the DNA coding sequence coded for
such proteins is prohibitively time consuming.
30   One prior art approach, now often followed,
utilizes chemically synthesized DNA in conjunction
with enzymatically prepared cDNA to produce a
gene which instructs production of mature hormone
in bacteria. Human growth hormone (HGH) is a
35 protein of 191 amino acids, its precursor having
an additional 26 amino acid "pre" portion. cDNA

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encoding the precursor was enzymatically prepared
from mRNA isolated from human pituitary tissue.
The first useful cleavage site of the cDNA occurs
at the site encoding amino acid residues 23-24 of
5   HGH. Treatment of the cDNA with restriction
endonuclease Hae III gives a DNA fragment of
551 base pairs which includes coding sequences for
amino acids 24-191 of HGH. A gene fragment
having coding sequences for residues 1-23 of HGH
10   (and an initiation codon) was chemically synthesized.
The two DNA fragments were combined to form a
synthetic-natural hybrid gene which when inserted
into a plasmid vector directed expression of
mature HGH in E. coli. (D. Goeddel, et al.,
15   Nature, Vol. 281, pp. 544-548 (October 1979)).
          Using a similar strategy of cleavage and
reconstriction of DNA for the mature protein,
R. Lawn et al., Nucleic Acids Research, Vol. 9,
No. 22, pp. 6103-6114 (1981), expressed mature
20   human albumin in E. coli.
          This general approach, however, requires
time consuming chemical synthesis of desired gene
fragments, cleavage of cDNA assuming the availa-
bility of useful cleavage sites and difficult
25   genetic construction of plasmids from DNA fragments.
Furthermore, in both of the above examples, an
initiator methionine was left at the NH$_2$ -terminal.
The initiator methionines cannot practically be
removed since HGH and albumin also have methionines
30   located elsewhere in the sequence. Thus, removing
the initiator methionine by cayanogen bromide
cleavage, would result in cleavage at the other
methionines. This would result in a protein split
into cleaved fragments. Both the HGH and albumin
35   produced by the above approach are "mature"
proteins which start with methionines. Hence they

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are not "real" mature proteins.

 The prior art approaches set forth above illustrate that a major and nearly universal problem in producing mature proteins is the con-
5 struction of the actual genetic material to be inserted into transfer vectors. Procedures exist for preparing cDNA from mRNA isolated from mammalian or other higher order animal tissue, but mammalian and higher order animal proteins are
10 most often expressed as precursors and subsequently processed into the mature protein in cells of origin. The prior art has identified E. coli and yeast as microorganisms capable of processing precursors containing the "pre" portion, but this
15 class of precursors excludes many of precursors of interest. The prior art thus has not identified a microorganism suitable for cloning mammalian and higher order animal genes which is capable of processing to mature proteins precursors of
20 greatest interest. The prior art approaches attempt to solve the problem by constructing genes that code for mature protein. However, although procedures now exist for identifying nucleotide coding sequences for mature proteins, chemical
25 synthesis of DNA sequences encoding mature proteins or fragments thereof for use in hybrid genes is costly and time consuming, often prohibitively so.

BRIEF DESCRIPTION OF THE FIGURES

30 FIG. 1 shows inferred protein cleavage sites within the precursor of yeast α-factor, where "K" designates lysine and "R" designates arginine amino acid residues.

 FIG. 2 shows the cDNA sequence encoding
35 preproparathyroid hormone and the unique Pvu II and Hinf 1 cleavage sites.

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FIG. 3 shows certain portions of the
nucleotide sequence of the pYEM-1 plasmid.

SUMMARY OF THE INVENTION

In the present invention, a method is
5   disclosed for producing protein in yeast transformed
to express a corresponding precursor containing
a pair or triplet of basic amino acid residues
located proximally and/or distally adjacent to the
protein portion of the precursor sequence comprising
10   proteolytic processing by the yeast of the
precursor at the site of such pairs or triplets
of basic amino acid residues. The method comprises
proteolytic processing by transformed yeast which
contains an endopeptidase, designated herein as a
15   trypsin-like enzyme or enzymes. The trypsin-like
enzyme or enzymes proteolytically process the
precursor at the site of such pairs or triplets
of basic amino acid residues by cleaving at the
distal side of such pairs or triplets. The
20   method further comprises proteolytic processing
by transformed yeast that contains an exopeptidase,
designated herein a carboxypeptidase-B-like
enzyme or enzymes. The carboxypeptidase-B-like
enzyme or enzymes proteolytically process the
25   precursor at the site of such pairs or triplets
of basic amino acid residues by degrading such
pairs or triplets of basic amino acid residues
remaining distally adjacent to the protein portion
of the precursor sequence after the cleavage by
30   the trypsin-like enzyme or enzymes.

In the present invention, the above method
is further disclosed for proteolytic processing of
proto-proteins to mature proteins. Proto-proteins,
defined with greater specificity _infra_, consist
35   generally of precursor proteins in which the

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protein portion of the precursor sequence is
identical in structure to the mature protein except
for the abscence of the amino terminal and the
carboxyl terminal in the precursor sequence. The
5   above method is also disclosed for proteolytic
processing of certain non-proto-proteins. For
example, the above method is disclosed for
proteolytic processing of preproinsulin or
proinsulin to mature insulin. The above method is
10   disclosed for producing mammalian insulin
generally as well as human, bovine, and porcine
insulin specifically. According to the method,
preprocalcitonin and procalcitonin may be
proteolytically processed by transformed yeast
15   to form mature calcitonin or a calcitonin relative
in the case of animal calcitonin generally and
human, bovine, and porcine calcitonin specifically.
        In the present invention, a recombinant
DNA plasmid transfer vector useful for transforming
20   yeast comprising a DNA sequence comprising the
preproparathyroid gene cDNA sequence is disclosed
as well as the plasmid pYEM-1 and yeast transformed
by a plasmid comprising the above transfer vector
and yeast transformed by the plasmid pYEM-1.
25   DESCRIPTION OF THE SPECIFIC EMBODIMENT
        Proto-proteins may consist of precursors
for which DNA and mRNA encoding the precursors
naturally occur in animals. This type of
proto-protein is designated source natural
30   proto-proteins. Proto-proteins may also consist
of precursors in which synthetic DNA encodes the
precursor. This type of proto-protein is designated
source synthetic proto-protein. For example, by
chemical synthesis, or alternatively by enzymatic
35   cleavage, rearrangement and subsequent fusion, DNA
can be synthesized so that the precursor which

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it encodes has the cleavage properties discussed
below. Production of mature protein might be
enhanced by transforming yeast with synthetic DNA
encoded for a precursor having repetitive sequences
5   of the mature protein, each sequence being flanked
by appropriate cleavage sites.

Source natural proto-proteins are illustrated
by, but not limited to, certain hormone precursors,
including preproparathyroid (J. Habener & J. Potts,
10 The New England Journal of Medicine (Second Part),
Vol. 299, No. 12, pp. 635-643 (Sept. 1978)),
preprosomatostatin (P. Hobart, et al., Nature,
Vol. 288, pp. 137-139 (November 1980)), AVP-NpII
precursor to arginine vasopressin and its
15 corresponding neurophysin (H. Land, et al., Nature,
Vol. 295, pp. 299-303 (January 1982)), cortitropin
B-lipotropin precursor to corticotopin (ACTH)
and B-lipotropin (B-LPH) (S. Nakanishi, et al.,
Nature, Vol. 278, pp. 423-427 (March 1979)),
20 preproglucagon (P. Lund, et al., Pro. Natl. Acad.
Sci. USA, Vol. 79, pp. 345-349 (January 1982)),
and pro-opiomelanocortin (POMC) precursor to
B-endorphin and Met- and Leu-enkephalin precursor
(M. Comb, et al., Nature, Vol. 295, pp. 663-666,
25 (February 1982)).

Source natural proto-proteins are also
illustrated by melittin precursor (G. Suchanek,
et al., Eur. J. Biochemistry, Vol. 60, pp. 309-315
(1975); G. Suchanek, et al., Proc. Natl. Acad.
30 Sci. USA, Vol. 75, pp. 701-704 (1978)) and serum
albumin precursors (R. Lawn, et al., Nucleic Acids
Research, Vol 9, No.22, pp. 6103-6114 (1981)).

As reported in the above citations, these
precursors contain within their sequence at least
35 one mature protein sequence. Where there is a
single mature protein sequence contained in the
precursor it is flanked proximally by a pair or

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triplet of basic amino acid residues consisting
of lysine and/or arginine and is flanked distally
by either the carboxyl-terminal of the precursor
or a pair or triplet of basic amino acid residues

5   lysine and/or arginine. If there are several mature
protein sequences contained in the precursor, at
least one of the mature protein sequences is
flanked proximally by a pair or triplet of such
basic amino acid residues and is flanked distally

10   by either the carboxyl-terminal of the precursor
or a pair or triplet of such basic amino acid
residues. Any precursor protein falling within
this description is defined herein as a proto-protein,
whether it be source natural or source synthetic.

15   As reported in the above citations in
connection with observing the production of mature
proteins in mammals and other higher order animals,
the cleavage site located on the distal side of
a pair or triplet of such basic amino acid residues

20   is readily attacked by endopeptidases with
trypsin-like activity. After endopeptidase
cleavage, any residual basic residues remaining
adjacent to and on the distal side of the mature
protein are susceptible to degrading, i.e.

25   selective removal, by exopeptidases with activity
resembling that of carboxypeptidase-B.
Thus, for example, in preproparathyroid
hormone the mature protein is flanked proximally
by the basic triplet lysine-lysine-arginine and is

30   flanked distally by the carboxyl-terminal of the
precursor. A single cleavage by a trypsin-like
enzyme is sufficient to produce the mature hormone.
In other proteins such as the glucagon precursor,
two mature glucagon proteins are flanked both

35   proximally and distally by a basic pair lysine-
arginine. Combined cleavage by a trypsin-like

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enzyme and degradation of the resulting carboxyl-
terminal by a carboxypeptidase-B-like enzyme are
required to produce the mature proteins.

5   The method of the present invention comprises
preteolytic processing by yeast of proto-proteins
to mature proteins. In the method, transformed
yeast naturally containing a trypsin-like enzyme
or enzymes and a carboxypeptidase-B-like enzyme
or enzymes, proteolytically release mature proteins
10 from larger precursors. These enzymes will effectively
cleave and degrade proto-proteins to mature proteins.
This is confirmed by a trypsin-like cleavage,
discussed infra, of preproparathyroid hormone
yielding mature parathyroid hormone. This is
15 further confirmed by yeast processing its own
mating factor, α-factor. (T. Tanaka, et al.,
J. Biochemistry, Vol. 82, pp. 1681-1687 (1977)).
As shown in FIG. 1, the nucleotide sequence of
α-factor shows that yeast naturally expresses a
20 precursor containing four distinct codings for
mature α-factor. Three of the four α-factors
in the precursor are flanked distally by a pair
of basic amino acids residues. A trypsin-like
cleavage in combination with a carboxypeptidase-
25 B-like degrading naturally yields correctly
processed C-termini for these three.α-factors.
After a trypsin-like cleavage, N-termini of the
four α-factors are flanked proximally by a series
of several glutamic acid and alanine amino acid
30 residues. These latter residues are in turn removed
by an aminopeptidase. The foregoing natural
endopeptidase and exopeptidase activity in yeast
in combination with the virtual uniform presence of
pairs and triplets of lysine and/or arginine
35 flanking mature hormone sequences in proto-proteins
underlies the present invention.

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Although preproinsulin and proinsulin containing
disulfide bonds are not proto-proteins as defined
herein they will nevertheless undergo proteolytic
processing in yeast transformed to express the
5   preproinsulin or proinsulin. A pair or triplet of
basic amino acid residues are located distally
and/or proximally adjacent to the insulin-A-
chain and the insulin-B-chain portions of the
sequence which constitute the protein portion of
10   the precursor preproinsulin and proinsulin
sequence. The requisite disulfide bonds between
the insulin-A-chain portion of the sequence and
the insulin-B-chain portion of the sequence will
be formed in yeast. (cf. the numerous examples
15   of disulfide bond formation in yeast disclosed in
M. Dayhoff, Atlas of Protein Sequence and Structure,
Vol. 5 and Supplements 1, 2 & 3 (National Biomedical
Research Foundation, Georgetown University Medical
Center, Washington, D.C. 20007 (1972, 1973, 1976,
20   and 1981))). Proteolytic processing at the site
of such pairs or triplets of basic amino acid
residues will yield mature insulin from preproinsulin
or proinsulin containing the disulfide bonds.
In the absence of disulfide bond formation
25   between the insulin-A-chain portion of the sequence
and the insulin-B-chain portion of the sequence,
proteolytic processing will yield insulin-A-chain
and insulin-B-chain, which may be caused in turn
to attach to one another by disulfide bonds by
30   conventional means to form mature insulin. In
this case, the insulin-A-chain and insulin-B-chain
may be considered mature proteins and preproinsulin
and proinsulin without disulfide bonds may be
considered a proto-protein according to the above
35   discussion of proto-proteins.

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          Mature calcitonin contains disulfide bonds
     between the cysteines located at positions 1 and
     7 of the sequence, contains a carbohydrate attached
     at the sequence at position 3, and the proline
5   at position 32 has been amidated to pro-amide
     while the glycine at position 33 has been removed.
     Preprocalcitonin and procalcitonin will contain
     the requisite disulfide bonds. (cf. the numerous
     examples of disulfide bond formation in yeast as
10   disclosed in Dayhoff, supra). A carbohydrate
     will be attached at position 3 in calcitonin.
     Preprocalcitonin and procalcitonin will undergo
     proteolytic processing in yeast transformed to express
     the preprocalcitonin or procalcitonin. A pair
15   of basic amino residues are located proximally
     adjacent to the 33 amino acid sequence, while a
     triplet is located distally adjacent to the 33
     amino acid sequence. It is expected that
     amidation of the proline located at 32 will
20   occur in yeast after the cleavage distall to and
     degradation of the triplet. (cf. numerous examples
     of amidation in yeast as disclosed by Dayhoff,
     supra). In the event that a carbohydrate
     differing from the carbohydrate of mature calcitonin
25   is formed by the yeast, the calcitonin relative
     containing the differing carbohydrate may be
     converted to mature calcitonin by conventional
     means. In the event that amidation following
     cleavage and degradation is suppressed, the
30   calcitonin relative lacking the amidation may also
     be converted to mature calcitonin by conventional
     means.
          By reverse transcription, cDNA can be
     prepared encoding any proto-protein of interest by
35   isolating mRNA from tissues expressing the protein.
     Although many hormone and other protein genes have

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already been cloned in E. coli, yeast has heretofore
not been the host of choice. cDNA not previously
cloned in yeast can be rendered compatible with a
yeast host by proper codon selection (J. Bennetzen &

5   B. Hall, J. Bio Chem., Vol. 257, pp. 3026 (1982))
and by site specific mutagenesis of the cDNA
(G. Simmons, et al., Nucleic Acid Research,
Vol. 10, pp. 821 (1982)).

        Thus, one of the fundamental problems with

10   producing useful mature proteins by recombinant
DNA techniques has been simplified in the case of
mature proteins derived from proto-proteins.
cDNA, although readily available for most proteins
by reverse transcription of mRNA isolated from

15   animal tissue, will express the precursor of the
mature protein. Yeast, but not E. coli, has the
requisite enzymes to process expressed proto-proteins,
preproinsulin, or proinsulin to mature protein or
insulin.

20   EXPERIMENTAL
        In order to demonstrate the present invention,
the following experiment was carried out.
        The plasmid YEp-13 was obtained from
Dr. Steven Henekoff, Fred Hutchinston, Dept. of

25   Developmental Biology, Seattle, Washington, and can
be constructed according to J. Broach, et al.,
Gene, Vol. 8, pp. 121-133, (1979). The gene which
encodes yeast alcohol dehydrogenase 1 was modified
according to Hitzelman, et al., Nature (London),

30   Vol. 293, pp. 717-722 (1981), allowing the
isolation of the transcription signals. These
sequences, including the cloning site, were
provided by Dr. G. Ammera. The plasmid YEp-13
was modified so that the tet^R gene of YEp-13 was

35   interrupted at the Bam Hl site with the yeast alcohol
dehydrogenase 1 gene promotor and RNA polymerase

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stop sequences. A Hind III site between the
latter two elements provided the cloning site.
These modifications of plasmid YEp-13 were
accomplished by methods set forth generally in
5   U.S. Patent 4,237,224, supra, and Methods of
Enzymology, Vols. 65 and 68 wherein such methods
are reviewed.

The cDNA sequence coding bovine prepropara-
thyroid hormone, shown in FIG. 2 and further
10   described in B. Kemper, et al., Hormonal Control
of Calcium Metabolism (Ed. by D. Cohn, et al.,
published Excerpta Medica at Amsterdam, Oxford,
and Princeton 1981) at pp. 19, was obtained from
Dr. Byron Kemper, Department of Physiology and
15   Biophysics and School of Basic Medical Sciences,
University of Illinois-Urbana. This cDNA sequence
was restricted with the enzymes PVU II and Hinf 1
at the sites shown in FIG.2. These enzymes were
obtained from New England Biolaboratories, Beverly
20   MA. The Hinf 1 site shown in FIG. 2 was filled with
nucleotides using the enzyme DNA polymerase 1
(the large fragment) which was obtained from
New England Nuclear, Boston, MA. This modified
sequence was then blunt-end ligated to Hind III linkers
25   and restricted with the enzyme Hind III. The
Hind III linkers and Hind III enzyme were obtained
from New England Biolaboratories, supra. The
resulting DNA fragment was then ligated into the
Hind III site of the modified plasmid YEp-13
30   forming a novel plasmid. This plasmid was designated
pYEM-1. FIG. 3 shows certain portions of the
nucleotide sequence of pYEM-1. The foregoing
construction of pYEM-1 was accomplished by methods
set forth generally in U.S. Patent No. 4,237,224,
35   supra, the BLR M13 handbook, and Methods of
Enzymology, Vols. 65 and 68 wherein such methods are

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reviewed.

          After constructing pYEM-1, yeast cells
were transformed with the plasmid using the methods
of Beggs, Nature (London), Vol. 275, pp. 104-109
5   (1978) and Hinnen, et al., Proc. Natl. Acad. of
Sci. USA, Vol. 75, pp. 1929-1933 (1978). Because
pYEM-1 has the yeast leu 2 gene, the use of a
leu 2 negative strain of yeast was used in the
transformation for the purposes of selecting
10   successful transformants. Yeast strain, X1069-2D,
a strain of Saccharomyces cerevisiae defective
in leu-2 function, was obtained from the Yeast
Genetic Stock Center, Univ. of Califorinia-
Berekeley.
15   Of course any other defective yeast strain,
including strains within Saccharomyces pombe and
other species, could be used. All that is required
is that a complementation system be established
between the yeast strain and the cloning/expression
20   vector and that the vector be stably maintained
in yeast. For example, a Trp 1 strain could be
used if the Trp 1 gene was on the vector. To
date, several stable transformation systems have
been described. (A. Hinnen and B. Meyhack,
25   Current Topics in Microbiology and Immunology,
Vol. 96, pp. 101-117 (1981); C. Hollenberg,
Current Topics in Microbiology and Immunology,
Vol. 96, pp. 119-144 (1981)).
          The transformed yeast cells containing
30   plasmid pYEM-1 were grown in a leucine deficient
media containing 5% glucose, yeast extract,
yeast nitrogen base and other nutrients suitable
for yeast strain X1069-2D. After 24 hours of
growth at 30°C, the media was collected and the
35   yeast cells lysed. Bioassay was performed
according to conventional techniques and PTH

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radioimmunoassay was performed using Immuno
Nuclear Corporation (Stillwater, MN) assays
specific to the N-terminal, mid-molecule, and
C-terminal regions of parathyroid hormone. The
5   following table shows that both immunologically
cross-reactive parathyroid hormone and biologically
active parathyroid hormone is being produced in
yeast.

10   TABLE

	PTH N-terminal RIA*	PTH Mid-molecule region RIA*	PTH C-terminal RIA*	Bioassay*
Cell lysate				
pYEM-1	16	16	16	10
control	0	0	0	0
Media				
pYEM-1	2	2	2	0.015
control	0	0	0	0

20   *expressed in nanomoles/ml

To confirm that correct processing had
occurred, 50 ml of culture was prepared in which
the parathyroid hormone producing yeast were
25   grown in media containing ^{35}S methionine
(80 μ ci/ml). After an overnight growth the
cells were removed by centrifugation. The media
was then incubated with specific N-terminal
parathyroid hormone antibody. After two hours
30   the antibody-antigen complex was recovered by
centrifugation and washed three times with new
media followed by an ether wash. This complex
contained about 7,000 cpm of ^{35}S methionine
incorporated into protein after TCA precipitation.
35   This mixture was applied to a Beckman 890D

sequencer according to the methods of Mahoney
and Nute, Biochem. Vol. 19, pp. 4436 (1980) and
subsequently degraded 40 cycles. Sequence analysis
demonstrated that the ^{35}S methionine was all

5   contained in cycles number 8 and 18. In mature
PTH, methionine appears only at positions
8 and 18 in the sequence. If prepproparathyroid
hormone expressed by the yeast was left unprocessed,
we would expect ^{35}S methionine in cycles 1, 2,

10   7, 11, 14, 49, and 59 reflecting the appearance
of methionine at positions -31, -30, -25, -21,
-18, +8, +18 in the prepproparathyroid sequence.

       The novel microorganism yeast strain
X1069-2D transformed by novel plasmid pYEM-1,

15   designated X1069-2D-pYEM-1, was placed on permanent
deposit in the Northern Regional Research Center,
U.S. Dept. of Agriculture, Peoria, Illinois
61604 on September 8, 1982. The NRRL number for
X1069-2D-pYEM-1 is Y-15153. The plasmid pYEM-1

20   and the transfer vector contained therein may be
removed from this novel yeast strain by known
means.

       While the invention has been described in
connection with a specific embodiment thereof, it

25   will be understood that it is capable of further
modifications and this application is intended to
cover any variations uses, or adaptations of the
invention within the scope of the appended claims.

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CLAIMS

1.   A method for producing protein in yeast
transformed to express a corresponding precursor
containing a pair or triplet of basic amino acid
residues located proximally and/or distally adjacent
to the protein portion of .the precursor sequence,
comprising proteolytic processing by the yeast
of the precursor at the site of such pairs or
triplets of basic amino acid residues.

2.   The method of claim 1 wherein the proteolytic
processing by yeast of the precursor at the site
of such pairs or triplets of basic amino acid
residues comprises cleaving, by a trypsin-like
enzyme or enzymes present in the transformed yeast,
at the distal side of such pairs or triplets of
basic amino acid residues.

3.   The method of claim 2 wherein the proteolytic
processing by yeast of the precursor at the site
of such pairs or triplets of basic amino acid
residues further comprises degrading, by a carboxy-
peptidase-B-like enzyme or enzymes present in the
transformed yeast, of any such pairs or triplets
of basic amino assay residues remaining distally
adjacent to the protein portion of the precursor
sequence after the cleavage by the trypsin-like
enzyme or enzymes.

4.   The method of claim 1 wherein the corresponding
precursor is a proto-protein.

5.   The method of claim 4 wherein the proto-
protein is source synthetic proto-protein.

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6.   The method of claim 4 wherein the proto-
protein is source natural proto-protein.

7.   The method of claim 6 wherein the source
natural proto-protein is bovine preproparathyroid
hormone.

8.   The method of claim 1 wherein the protein
is mammalian insulin and the corresponding precursor
is mammalian preproinsulin or proinsulin.

9.   The method of claim 8 wherein the
mammalian insulin and mammalian preproinsulin
or mammalian proinsulin are members respectively
of the group consisting of human insulin and
human preproinsulin or human proinsulin,
bovine insulin and bovine preproinsulin or
bovine proinsulin, and porcine insulin and porcine
preproinsulin or porcine proinsulin.

10.   The method of claim 1 wherein the protein
is animal calcitonin or an animal calcitonin
relative and the precursor is animal preprocalcitonin
or animal procalcitonin.

11.   The method of claim 10 wherein the animal
calcitonin or animal calcitonin relative and the
animal preprocalcitonin or animal procalcitonin
are members respectively of the group consisting
of human calcitonin or human calcitonin relative
and human preprocalcitonin or human procalcitonin,
bovine calcitonin or bovine calcitonin relative
and bovine preprocalcitonin or bovine procalcitonin,
and porcine calcitonin or porcine calcitonin
relative and porcine preprocalcitonin or pro-
calcitonin.

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12.   The method of claim 1 wherein the yeast
is Saccharomyces cerevisiae or Saccharomyces
pombe.

13.   The method of claim 12 wherein the yeast
is Saccharomyces cerevisiae.

14.   A recombinant DNA plasmid transfer vector
useful for transforming yeast comprising a
DNA sequence comprising the preproparathyroid
gene cDNA sequence.

15.   The plasmid pYEM-1

16.   Yeast transformed by a plasmid comprising
the transfer vector of claim 14.

17.   Yeast transformed by the plasmid of
claim 15.
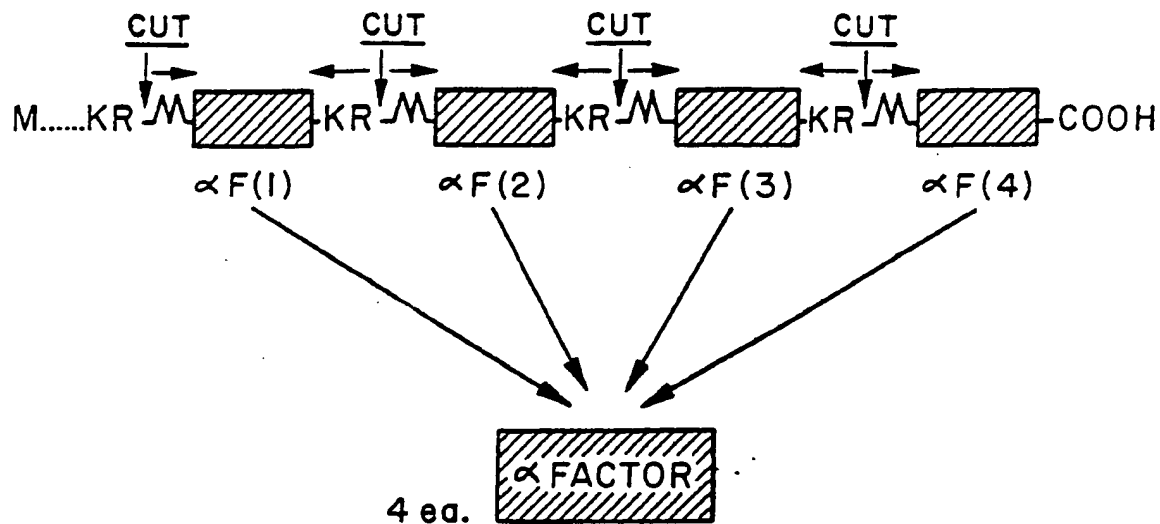
Fig 1
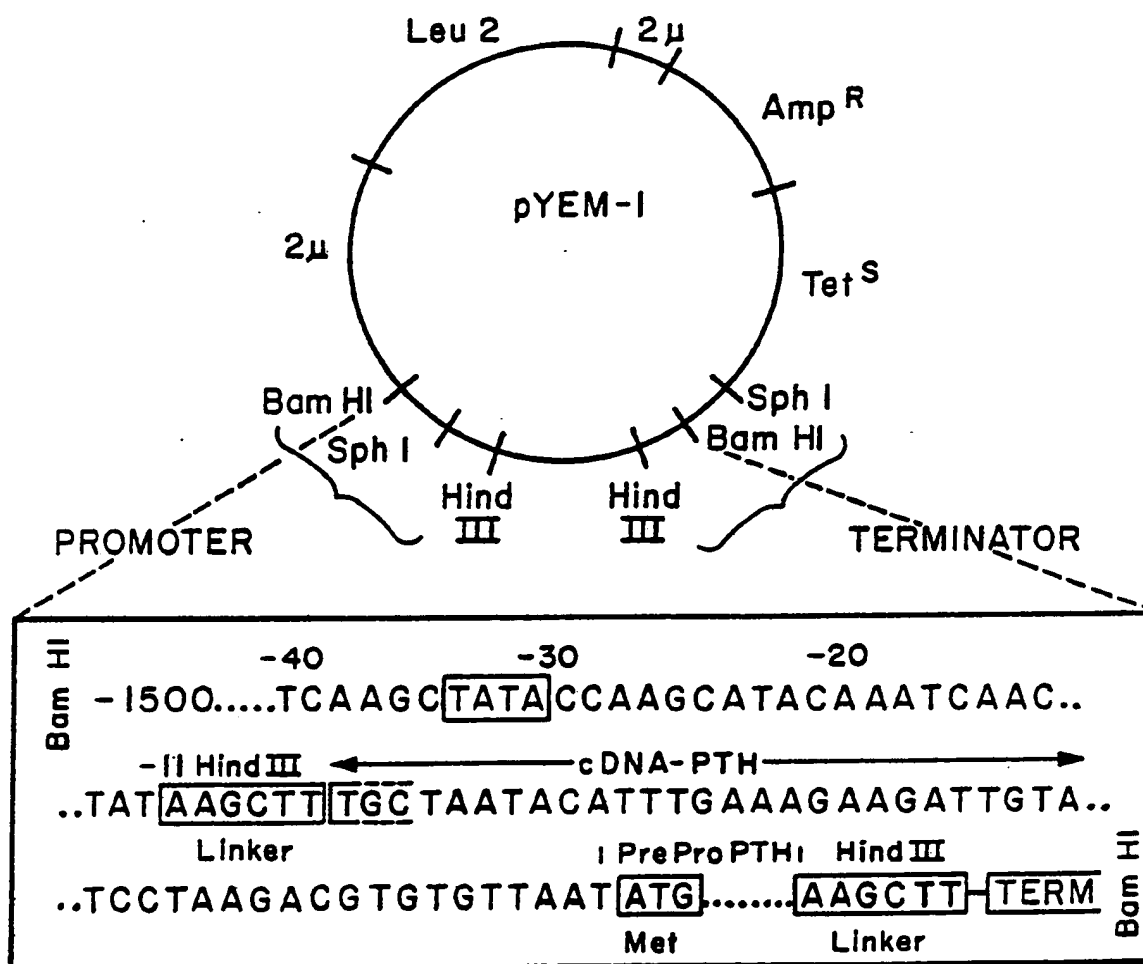
Fig 3

PvuII Cleavage Site C/T

5' G GGG GGG GGG GGG GTT TTA TCA GCC TTC TCA GGT TTA CTC AAC TTT GAG AAA GCA TCA GCT AAT ACA TTT
          10   20   30   40   50   60   70

  met met ser ala lys asp met val lys val met ile val met leu
GAA AGA AGA TTG TAT CCT AAG ACG TGT GTT AAT ATG ATG TCT GCA AAA GAC ATG GTT AAG GTA ATG ATC GTC ATG CTT
80   90   100   110   120   130   140   150

ala ile cys phe leu ala arg ser asp gly lys ser val lys lys arg ala val ser glu ile gln phe met his asn leu
GCC ATC TGT TTT CTT GCA AGA TCA GAT GGG AAG TCT GTT AAG AAG AGA GCT GTG AGT GAA ATA CAG TTT ATG CAT AAC CTG
160   170   180   190   200   210   220   230

gly lys his leu ser ser met gly arg val glu trp leu arg lys lys leu gln asp val his asn phe val ala leu gly
GGC AAA CAT CTG AGC ATG GGA AGA GTG GAA TGG CTA CGG AAA AAG CTA CAG GAT GTG CAC AAC TTT GTT GCC CTT GGA
240   250   260   270   280   290   300   310

ala ser ile ala tyr arg asp gly ser ser gln his arg arg pro arg lys glu asp asn val leu val glu ser his gln
GCT TCT ATA GCT TAC AGA GAT GGT AGT TCC CAG CAT AGA AGA CCT CGA AAA GAA GAC AAT GTC CTG GTT GAG AGC CAT CAG
320   330   340   350   360   370   380   390

lys ser leu gly glu ala asp lys ala asp val leu ile lys pro gln stop
AAA AGT CTT GGA GAA GCA GAC AAA GCT GAT GTG GAT TTA ATT AAA GCT AAA CCC CAG TGA AAA CAG ATA TGA TCA GAT
400   410   420   430   440   450   460   470

CAC TGT TCT AGA CAG CAT AGG GCA ACA ATA TTA CAT GCT GCT AAT GTG TTC ACC TTC TAT TAA GTG CCA GTA GTT CTA TGA
480   490   500   510   520   530   540   550

Hinf Cleavage Site G/A

CCA ACC TTT ATT GCT AGC TGT GAT ACC TAC AAT TTT AAT TGA GTA TTT TGA TTC TAC TTT CAT CTA AGA GCT CTT
560   570   580   590   600   610   620   630

TTA ATA ATT CTA TTG ATT CCA AAT AAA TGA TTA AGT TAA GTA AAA AAA AAA AAA AAA AAA AAA
640   650   660   670   680   690   700   710

AAA AAA AAA AAA AAA CCC CCC CCC CCC CCC CCC CCC 3'
720   730   740   750   760

Fig 2

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.³ C12P21/00,C12N15/00,1/18,1/00
U.S. CL. 435/68,172,256,317

II. FIELDS SEARCHED

Minimum Documentation Searched *	
Classification System	Classification Symbols
U.S.	435/68,172,256,317

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

CHEMICAL ABSTRACTS FILES 308,309,310,320 and 311
BIOSIS FILES 5,55 and 255

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N, WILLIAMSON, GENETIC ENGINEERING 4 ACADEMIC PRESS PP108-125,1983.	1-17
A	N, WALTON, RECOMBINANT DNA ELSEVIER SCIENTIFIC PUBLISHING CO., PP.185-197 and 213-227.	1-17
A	GB, A 2068969 A, PUBLISHED 19 AUGUST 1981.	1-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search *	Date of Mailing of this International Search Report *
25 NOVEMBER 1983	06 DEC. 1983
International Searching Authority *	Signature of Authorized Officer [11]
ISA/US	ALVIN E. TANENHOLTZ

The Amino-Acid Sequence of the Amino-Terminal 37 Residues of Human Parathyroid Hormone

(high-sensitivity automated Edman degradation/radio-iodination/peptide synthesis/immunoreactivity)

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ABSTRACT   The sequence of the amino-terminal 37 residues of human parathyroid hormone has been established. The hormone used in these studies was isolated in highly purified form from parathyroid adenomata and was subjected to automated degradation in a Beckman sequencer. A high-sensitivity sequencing procedure employing ³⁵S-labeled phenylisothiocyanate of high specific activity as the coupling agent was used. The sequence obtained differs from that of bovine parathyroid hormone in three of the first 37 positions, and from that of porcine parathyroid hormone in two positions. A single human-specific residue was found (asparagine 16). The sequence obtained differs at three positions (22, 28, and 30) from the structure for human parathyroid hormone reported recently by Brewer et al. [(1972) *Proc. Nat. Acad. Sci. USA* 69, 3585–3588] and synthesized by Andreatta et al. [(1973) *Helv. Chim. Acta*, 56, 470–473] We have carefully reviewed our data, reported here in detail, on the sequence positions in dispute. We must conclude, on the basis of all available data, that the structure that we propose is the correct structure. The objective resolution of these discrepancies in structural analysis through further chemical and immunochemical studies is important, since synthesis of human parathyroid hormone, in which there is widespread interest for physiological and clinical studies, must be based on the correct sequence of the human hormone if the peptide is to be genuinely useful.

Substantial advances have been made in recent years in our knowledge of parathyroid hormones, through studies of primary structure (1–3), structural requirements for biological activity (4, 5), biosynthesis (6–8), and metabolism (9–14). Most of these studies, including the development and application of radioimmunoassays capable of measuring plasma parathyroid hormone levels in man, have depended directly or indirectly upon the use of the bovine and porcine hormones. Purified human parathyroid hormone (HPTH), on the other hand, has been available in microgram quantities, sufficient only for limited studies of its chemical and immonological properties (15).

Recent improvements in extraction and isolation techniques, and the development of high-sensitivity methods for peptide sequence analysis have permitted us to determine the amino-acid sequence of the amino-terminal biologically active portion of HPTH (Fig. 1).

After the submission for publication in abstract form of our findings for the N-terminal 31 residues of HPTH (24), the report of Brewer et al. (29) of their own independent struc-

tural studies on HPTH was published. Marked discrepancies between the two structures, which differ in three of the first 30 residues, have prompted us to reexamine our data for each cycle of the several degradations performed with the phenylisothiocyanate method.

We now report in full the strategy and methods used in our sequence analysis as well as the quantitative aspects of the results and discuss the nature, implications, and possible approaches to resolution of the differences between the findings of Brewer et al. (29) and ourselves concerning the sequence of the amino-terminal portion of human parathyroid hormone.

MATERIALS AND METHODS

The HPTH used in these studies was extracted from 500 g of pooled human adenoma tissue by use of 88% phenol, followed by treatment with 6% NaCl and precipitation with trichloroacetic acid (15, 16). The hormone was further purified by gel filtration on Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) and ion-exchange chromatography on carboxymethyl-cellulose (Whatman CM-52; Reeve Angel Co., Summit, N.J.) (16). Hormone purification was monitored by radioimmunoassay (11).

Automated Edman degradations were performed on the Beckman model 890 sequencer (Beckman Instruments, Palo Alto, Calif.) using the single-coupling, double-cleavage method of Edman and Begg (17), and other procedures recently described (18). Manual Edman degradations were performed as previously described (19). Reagents and solvents were obtained from Beckman Instruments. ³⁵S-Labeled phenylisothiocyanate was obtained from Amersham/Searle (Arlington Heights, Ill.).

The phenylthiohydantoin (PTH) derivatives were identified by thin-layer chromatography (TLC) on silica gel plates (Analtech, Inc., Newark, Del.) (17, 20) and by gas-liquid chromatography (21) using a two-column system (10% DC-560 and 1.5% AN-600). PTH-histidine was identified by the Pauly reaction (22) and PTH-arginine by the phenanthrenequinone reaction (23). Quantitative yields of the PTH-amino-acid derivatives at each cycle of degradation were determined by comparison with known standards on gas-liquid chromatography. The [³⁵S]PTH-amino acids were separated by TLC; the radioactive spots were identified by autoradiography and quantitated in a Packard model 3375 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Mono-iodohistidine (MIH) and di-iodohistidine (DIH) were synthesized by the method of Brunnings (25) using the modifications of Savoie et al. (26). The phenylthiohydantoin

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Abbreviations: HPTH, human parathyroid hormone: PTH, phenylthiohydantoin; TLC, thin-layer chromatography; MIH, mono-iodohistidine; DIH, di-iodohistidine.

TABLE 1. *Automated Edman degradation of native HPTH*

PTH-amino acids			PTH-amino acids		
Cycle	Found	Yield (nmol)	Cycle	Found	Yield (nmol)
1	Ser	80.0	21	Val	23.8
2	Val	130.2	22	Glu†	10.5
				Thr	7.3
3	Ser	85.3	23	Trp	10.1
4	Glu	71.4	24	Leu	18.7
5	Ile	108.6	25	Arg	13.0
6	Glu*	20.7	26	Lys	14.6
	Gln	35.0			
7	Leu	97.5	27	Lys	15.9
8	Met	51.5	28	Leu	11.0
9	His	42.3	29	Glu*	3.1
				Gln	2.8
10	Asp*	30.6	30	Asp	4.5
	Asn	20.1			
11	Leu	97.5	31	Val	9.1
12	Gly	47.5	32	Ser†	0.7
13	Lys	62.1	33	Asp*	2.7
				Asn	‡
14	His	27.6	34	Phe	3.0
15	Leu	55.5	35	Val	3.0
16	Asp*	17.7	36	Ala	1.4
	Asn	18.1			
17	Ser	43.0	37	Leu	2.1
18	Met	34.8	38	—	—
19	Glu	20.2	39	Ala	1.2
20	Arg	16.4	40	—	—

* Partial deamidation during the conversion reaction accounts for the presence of the free acid as well as the amide form at positions 6 and 29 (glutamines) and positions 10, 16, and 33 (asparagines).

† See *text* for discussion.

‡ TLC identification.

derivatives of MIH and DIH were prepared as described by Edman (27). PTH-MIH and PTH-DIH were separated from all other PTH-amino-acid derivatives by TLC in the solvent system *n*-butyl acetate:water:propionic acid:formamide (240:200:30:60). PTH-[^{125}I]MIH and PTH-[^{125}I]DIH were identified by cochromatography with their respective ^{127}I derivatives followed by autoradiography, and quantitated by counting in a Packard model 3001 gamma well spectrometer.

NH$_2$-SER-VAL-SER-GLU-ILE-GLN-LEU-MET-HIS-ASN-LEU-GLY-LYS-HIS-LEU-ASN
     1   2   3   4   5   6   7   8   9   10   11   12   13   14   15   16

SER 17
MET 18
GLU 19
ARG 20
VAL 21

...LEU-ALA-VAL-PHE-ASN-HIS-VAL-ASP-GLN-LEU-LYS-LYS-ARG-LEU-TRP-GLU
    37   36   35   34   33   32   31   30   29   28   27   26   25   24   23   22

FIG. 1. The amino-terminal 37 residues of human parathyroid hormone.

Cleavage of the hormone with cyanogen bromide (CNBr) was carried out in 70% formic acid for 12 hr, 20°, with a 100-fold molar excess of CNBr. Digestion with TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.) was performed in 0.2 M trimethylamine acetate buffer (pH 9.2) at 37°, for a period of 2 hr using an enzyme-to-substrate ratio of 1/100.

RESULTS

Purified HPTH (140 nmol) was subjected to automated Edman degradation for 40 cycles. The PTH-amino acid derivatives identified at each cycle of this degradation, and their yields, are presented in Table 1. To illustrate repetitive yield these results are also plotted in Fig. 2.

As shown in Table 1, unique amino-acid assignments, and quantitation of the single residue identified, were possible at all but two of the first 37 cycles of this degradation. At cycle 22, evidence was obtained for two residues, threonine and glutamic acid. Since the quantitative recovery of both of these residues can be low, further experiments were performed prior to definitive assignment of position 22. At cycle 32, a rise in PTH-serine above background levels was observed. However, its yield (Table 1) was considerably below that expected, even for the labile phenylthiohydantoin derivative of serine (18). Although histidine is present at this position in porcine and bovine parathyroid hormones, this residue could not be detected either by the Pauly method or by a definite increase in radioactivity associated with [^{35}S]PTH-histidine at this cycle. However, since the overall yield at this stage of the degradation was near the detection limits for histidine by these methods, further experiments were performed prior to assignment of this position.

The presence of methionine at positions 8 and 18 of the native hormone accounted for both methionines found by amino-

FIG. 2. Yields of phenylthiohydantoin-amino acids obtained during automated degradation of native human parathyroid hormone. See Table 1 and *text*.
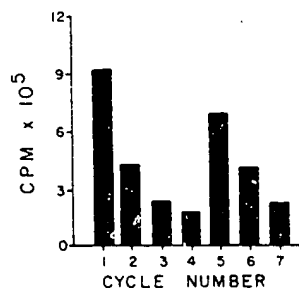
FIG. 3.   Release of radioactive (^{125}I-labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during automated degradation of ^{125}I-labeled human parathyroid hormone. The break in the horizontal axis is introduced to simplify the presentation. No significant release of histidine-associated radioactivity was seen in cycles 17-28. [^{125}I]Histidine was found at cycles 9, 14, and 32, indicating the presence of histidine at these positions. See *text*. Numbers on the ordinates are to be multiplied by the indicated factors to obtain the experimental values.

acid analysis (16). This indicated that cleavage of the human hormone with CNBr should result in generation of three principal peptides representing residues 1-8, 9-18, and 19-carboxyl terminus of the native hormone. HPTH (27 nmol) was cleaved with CNBr and the unfractionated peptide mixture subjected to 19 cycles of Edman degradation. The results are presented in Table 2. The expected three end-groups, Ser[1], His[9], and Glu[19] were identified at cycle one of the degradation. At cycle 4 of the degradation, corresponding to residues 4, 12, and 22 of the intact hormone, only PTH-Glu and PTH-Gly were observed in significant yield. No threonine was detected at this cycle. Therefore, glutamic acid was assigned as residue 22 of native HPTH. The significance of the finding of threonine in the amino-terminal degradation remains uncertain. As can be seen in Table 2, the results of the CNBr mixture analysis also provided complete confirmation of all residue assignments made on the basis of the amino-terminal degradation on intact HPTH.

Since the limited supply of purified HPTH excluded the use of conventional protein chemical methods for reexamination of position 32, an alternative radioactive micro-method was developed to permit detection of histidine residues. Purified HPTH (0.75 μg) was iodinated with ^{125}I by a modification of the Hunter-Greenwood procedure (28). Unlabeled bovine parathyroid hormone was then added as carrier and the mixture was degraded in the sequencer. At each cycle, the radioactivity migrating with PTH-[^{127}I]MIH and PTH-[^{127}I]DIH on TLC was determined. These data (Fig. 3) demonstrate

FIG. 4.   Release of radioactive (^{125}I-labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during degradation of a tryptic digest of iodinated HPTH. [^{125}I]Histidine was released at cycles 1 and 5. See *text*.

Cycle	CNBr1	CNBr2	CNBr3	Yield of PTH Derivative (nmol)
1	Ser[1]	His[9]	Glu[19]	Ser 18.3, His*, Gly 27.0
2	Val	Asn	Arg	Val 11.7, Asn 4.3, Arg†
3	Ser	Leu	Val	Ser 10.2, Leu 8.5, Val 18.4
4	Glu	Gly	Glu	Glu 4.5, Gly 3.1
5	Ile	Lys	Trp	Ile 10.2, Lys‡, Trp 58
6	Gln	His	Leu	Gln 4.9, His*, Leu 10.5
7	Leu	Leu	Arg	Leu 9.8, Arg†
8	Met§	Asn	Lys	Met 3.4, Asn‡, Lys‡
9	His	Ser	Lys	His*, Ser 4.2, Lys‡
10	Asn	—	Leu	Asn‡, Leu 7.5
11	Leu	—	Gln	Leu 1.4, Gln 4.6
12	Gly	—	Asp	Gly 0.9, Asp 2.3
13	—	—	Val	Val 4.2
14	—	—	—¶	—
15	—	—	Asn	Asn‡
16	—	—	Phe	Phe 2.6
17	—	—	Val	Val 4.5
18	—	—	Ala	Ala 2.0
19	—	—	Leu	Leu 2.0

* Identification by Pauly reaction.
† Identification by phenanthrenequinone reaction.
‡ Identification by thin-layer chromatography.
§ Presence of methionine at cycle 8 with the following four residues obtained at cycles 9-12 indicates that cleavage of the Met[8]-His[9] bond by cyanogen bromide was incomplete. Residues from this sequence, presumably representing the 1-18 peptide fragment, could not be detected subsequent to cycle 12.
¶ Histidine, subsequently found to occupy position 32 (see *text*) was not detected at the expected cycle (number 14) of this degradation.

the presence of histidine at cycle 32, and confirm the histidine at cycles 9 and 14.

To confirm these results further, and in particular to examine the differences between Brewer et al. (29) and ourselves concerning the nature of residue 28, iodinated HPTH was digested with trypsin and then subjected to Edman degradation for seven cycles. The PTH derivatives of [^{125}I]MIH and [^{125}I]DIH were found at cycles 1 and 5 of the degradation Fig. 4). The histidine at cycle 1 further confirmed the Lys[13]-His[14] sequence already determined. The finding of histidine at cycle 5 would be predicted on the basis of tryptic cleavage carboxyl to Lys[27], and therefore both supports the assignment of His[32] and argues against the report of Brewer et al. (29) that residue 28 is lysine.

DISCUSSION

The amino-terminal sequence we propose for HPTH differs from that of both the bovine and the porcine hormones. HPTH differs from bovine parathyroid hormone (Fig. 5) at positions 1, 7, and 16. The porcine and human hormones differ at positions 16 and 18. Asn[16] is the only unique residue found in the active region of human parathyroid hormone.

Unexpectedly, our structure differs at three positions from that recently proposed by Brewer et al. (29) for the amino-terminal 34 residues of HPTH. They report residue 22 to be glutamine, residue 28 to be lysine, and residue 30 to be leucine. If correct, all these changed residues would be unique to the human hormone. In contrast, we find residue 22 to be glutamic

FIG. 5. Comparison of the amino-terminal sequences of porcine, bovine, and human parathyroid hormones. The central continuous sequence is that of the human hormone (residues 1, 7, and 16). Residues differing in the bovine hormone are *stippled*; those differing in porcine parathyroid hormone (16 and 18) are *hatched*.

acid, residue 28 to be leucine, and residue 30 to be aspartic acid. The residues we have identified are identical with those at the corresponding positions in both the porcine and bovine hormones.

Since both groups have isolated the hormone from essentially similar sources, i.e., human adenoma tissue pooled from many centers, the possibility that there are two hormonal forms which differ as markedly as those of the two proposed sequences is highly unlikely.

We have carefully reexamined our data from both degradations with particular emphasis on the positions in question. In neither degradation was any glutamine observed at cycles corresponding to residue 22. Glutamine and asparagine can undergo deamidation during degradation. However, in both degradations (Tables 1 and 2) glutamine and asparagine residues were detected at cycles beyond position 22, making it implausible that the glutamic acid detected at position 22 was originally glutamine. At cycles corresponding to residues 28 and 30, leucine and aspartic acid were clearly identified by their predominant yields (Tables 1 and 2). The relative rise in yield of these residues above and subsequent fall to background levels is shown in Fig. 6.

In automated Edman degradation the phenomenon of increasing overlap, which tends to be cumulative from cycle to cycle, has been well documented (17, 18, 30). Edman and Begg have, however, found that use of a double-cleavage program can limit this overlap to relatively low levels (17) even in extended degradations. In our amino-terminal degradation, which employed such a double-cleavage program, overlap rose from 4.5% at cycle 12 to 14.5% at cycle 28. Brewer *et al.* reported quantitative data only at position 12; their data permit the calculation that there was a 32% overlap at this early phase of degradation. The natural increase of this already substantial overlap, particularly in view of their use of a single-cleavage program, would make assignment of repeating residues at later cycles of the degradation, such as the putative Lys[28] in a sequence Lys[26]-Lys[27]-Lys[28], particularly hazardous.

If, as proposed by Brewer *et al.* (29), residue 28 is lysine, tryptic digestion of [^{125}I]HPTH would lead to cleavage of the

FIG. 6. Yields of PTH-leucine at cycles 26–30 and of PTH-aspartic acid at cycles 28–32 obtained during automated degradation of native human parathyroid hormone. A sharp rise above background levels is seen at cycle 28 for leucine and at cycle 30 for aspartic acid. See *text.*

hormone carboxyl-terminal to residue 28. Therefore, the PTH derivatives of [^{125}I]MIH and [^{125}I]DIH corresponding to His[32] would be released at the fourth cycle of degradation, rather than the fifth. Clearly, however, histidine is released only at the first and fifth cycles (Fig. 4).

Ultimate resolution of the differences in the proposed structures must await further studies. However, a comparison of our results and methods with the published results and methods of Brewer *et al.* (29) leads us to conclude that our proposed structure based on a variety of approaches is more likely to be correct.

Tests of the biological and immunological properties of synthetic peptides corresponding to our structure and to the structure proposed by Brewer *et al.* (29) for the amino-terminal portion of human parathyroid hormone may prove helpful in objectively resolving the discrepancies in the structures proposed. The marked differences, which include a change in net charge of three within a sequence of nine residues, might affect biological activity. Even more likely is the possibility that such charge differences will result in clear-cut differences in immunoreactivity when the synthetic peptides based on the two proposed structures are each compared with native human parathyroid hormone in their ability to combine with antisera directed against the amino-terminal region (11).

If the structure of Brewer and associates is, as we believe, incorrect, use of antisera generated against the corresponding synthetic peptide for radioimmunoassay studies could confuse rather than aid attempts to more accurately measure HPTH or to understand the complex pattern of metabolism of parathyroid hormone (11). A preliminary immunoassay study based on the peptide of Brewer *et al.* (29) and Andreatta *et al.* (34) has already been published (35). Clearly it is extremely important to establish whether our sequence or that of Brewer *et al.* (29) represents the native HPTH structure before various laboratories embark on extensive immunological studies using synthetic HPTH peptide.

An amino-terminal tetratriacontapeptide based on the structure proposed here has been synthesized by the solid-phase method (31). Studies of the potency of this peptide as measured *in vitro* by activation of renal-cortical adenylate cyclase indicate that its activity is 1030 units/mg, closely equivalent, on a molar basis, to the potency of 350 units/mg (16) for native human parathyroid hormone in this assay. Assays *in vivo* using the chick hypercalcemia assay (32) indicate a potency of 7000 units/mg, an activity identical to that of the bovine peptide 1-34 (no native human parathyroid hormone was available for assay in this system).

The immunological activity of the synthetic peptide has been examined with several antisera directed against the amino-terminal region of parathyroid hormone. Tests against antiserum 199 (33) and GP-1 (11) indicated that reactivity on a molar basis of our synthetic peptide was identical qualitatively and quantitatively to that of the native human hormone. No details have been reported on the specific biological or immunological activity of the synthetic peptide of Andreatta et al. (34), whose structure was based on the structure reported by Brewer et al. (29). Comparisons based on detailed biological and immunological tests of the two synthetic peptides in various laboratories should be of considerable interest.

Our present findings carry several implications for the structural and comparative immunochemical studies of human parathyroid hormone. The sequence of HPTH we find is identical with that of either the bovine or porcine hormone at 36 of the first 37 residues; the changes found do not affect net charge and do not greatly alter physicochemical properties. Hence, although some improvements in detection of human parathyroid hormone might result from use of antisera directed against the amino-terminal sequence of the human hormone, the improvements, in our view, might not be large. In fact, the success encountered already in numerous laboratories in detection of the human hormone with immunoassays based on the bovine molecule is consistent with the overall chemical similarity found in the amino-terminal sequences of the three species of parathyroid hormone.

On the other hand, previous immunochemical and analytical evidence (11, 15, 16) indicates that more marked differences in structure between bovine and human hormones are likely to be found in the carboxyl-terminal region. Since a large carboxyl-terminal fragment appears to be the major form of immunoreactive parathyroid hormone in the human circulation (11), antisera that recognize the carboxyl end of the human hormone are most likely to significantly improve immunoassay sensitivity. Further sequence studies on HPTH, followed by synthesis of selected peptides from the carboxyl-terminal two-thirds of the molecule, may well result in antisera considerably more sensitive for detection of human parathyroid hormone.

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Isolation of Human Parathyroid Hormone†

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ABSTRACT: A pure preparation of human parathyroid hormone has been isolated using as starting material 500 g of pooled gland tissue removed at surgery. Following initial extraction of the acetone-dried parathyroid tissue by means of phenol, a trichloroacetic acid precipitate was obtained which contained 4% hormone by radioimmunoassay. Further purification of the initial CCl_3COOH precipitate was achieved by polyacrylamide gel filtration followed by a final step of ion-exchange chromatography. Repeat extractions of the original tissue residue provided an additional yield of hormone, although the hormone content of most of these CCl_3COOH preparations was lower. Additional ion-exchange chromatographic steps were needed for final purification of hormone derived from these repeat tissue extractions. The purified human hormone preparations from the various extractions were, however, found to be identical chemically and immunologically. An overall yield of 3.2 mg of purified hormone suitable for structural studies was obtained, sufficient for sequence analysis of the biologically active amino-terminal portion of the molecule. The biological activity by *in vitro* renal adenylyl cyclase assay is 350 units/mg, considerably lower than that of the hormone from the bovine species. The amino acid composition of human parathyroid hormone bears considerable resemblance to that of bovine and of porcine hormone. There are, however, a number of compositional differences which have not been accounted for in the sequence of the amino-terminal region. This indicates that when structural studies of the carboxyl-terminal portion are undertaken, they will reveal several sequence positions which are unique to the human hormone.

Evidence from several laboratories suggests that the state of parathyroid hormone in the circulation, especially in certain disease states, may be very complex (Habener *et al.*, 1971; Canterbury and Reiss, 1972; Segre *et al.*, 1972; Goldsmith *et al.*, 1973; Silverman and Yalow, 1973). Application of the radioimmunossay to measurement of parathyroid hormone in the peripheral circulation has demonstrated the presence of multiple fragments in addition to the intact hormone. The possible contribution of large precursor forms (Cohn *et al.*, 1972; Habener *et al.*, 1972) to the spectrum of immunoreactive hormone may further complicate this picture.

Attempts to clarify the nature of circulating human hormone and to ascertain the significance of the various hormonal fragments have thus far relied principally on radioimmunoassay systems and structure–function studies based on the bovine and porcine hormones. These problems have emphasized the need for knowledge of the chemical structure of human parathyroid hormone (HPTH).[1]

The structural analyses previously carried out on the hormone from bovine (Brewer and Ronan, 1970; Niall *et al.*, 1970) and porcine (O'Riordan *et al.*, 1971a) species benefitted from the availability of gland tissue obtained from slaughter-house sources. The study of human parathyroid hormone (Arnaud *et al.*, 1970; O'Riordan *et al.*, 1971b) has been limited by the extremely small quantities of available starting material: parathyroid tissue removed at surgery. Using this source, O'Riordan *et al.* (1971b) isolated 0.5 mg of purified hormone, sufficient for preliminary immunological and chemical characterization. It was observed, however, that throughout the isolation procedure the recoveries of hormone were considerably lower than those obtained during isolation of bovine or porcine hormone. These problems have stressed the need not only for efficient extraction and purification procedures, but also for the application of precise and economical methods for monitoring recoveries and purity throughout the isolation.

Recently, accumulation of sufficient quantities of human parathyroid tissue has permitted the resumption of efforts to characterize the human hormone, and, as a result, sequence analysis of the biologically active amino-terminal region has been reported by two groups of investigators (Brewer *et al.*, 1972; Niall *et al.*, 1974). To be described here is our procedure for the extraction and purification of hormone from 500 g of human gland tissue, using several modifications designed to improve recoveries. In the course of the work, compositional analyses of the hormone and an assessment of its biological activity have also been carried out.

The purified hormone obtained was sufficient for sequence determination of the amino-terminal 37 residues of the molecule (Niall *et al.*, 1974). Since the structural analysis of the entire carboxyl-terminal portion remains to be done, the information described in this report should be useful in planning and carrying out future hormone purifications when sufficient tissue again becomes available.

Materials and Methods

Parathyroid Tissue. The human parathyroid tissue used consisted predominantly of adenomas but also included hyperplastic tissue from patients with primary and secondary

[1] Abbreviations used are: HPTH, human parathyroid hormone; BPTH, bovine parathyroid hormone; PPTH, porcine parathyroid hormone; CMC, carboxymethylcellulose; MRC, Medical Research Council.

hyperparathyroidism. This material was obtained through the cooperation of a large number of medical centers in the United Kingdom, United States, and several European countries. The tissue was frozen on dry ice immediately upon surgical removal, and stored at −20° or below until used.

Extraction Procedure. The procedure followed for extraction of a crude hormone preparation from the pooled gland tissue is outlined in Figure 1. The tissue was first homogenized in acetone at −20° and filtered. The residue was defatted by homogenization in hexane, followed by filtration and washing with acetone, all at −20°. The resulting powder (AP, Figure 1) was kept overnight in an evacuated desiccator, and processed into a trichloroacetic acid precipitate (CCl₃COOH–HPTH) after extraction with phenol, by means of a procedure derived from that described originally by Aurbach (1959).

The acetone powder was suspended in 1 l. of 90% phenol containing 0.5% 2-mercaptoethanol. The suspension was diluted out with 5 l. of 20% acetic acid in acetone, 13 ml of 4 M sodium chloride was added and, after standing for 1.5 hr, the suspension was filtered in the presence of Celite.

The residue was saved and ultimately subjected to repeat extractions, as outlined in the Results section and in Figure 1. The filtrate was treated by gradual addition of ether and collection of the resulting precipitate (AE, Figure 1). The ether powder was resuspended in glacial acetic acid in the presence of cysteine-HCl, diluted with water, and centrifuged. The precipitate was saved for reprocessing later while the supernatant was made to 6% (w/v) with sodium chloride.

The precipitate formed upon addition of the salt was separated by centrifugation, resuspended, and again treated with sodium chloride. The combined salt supernatants (AS, Figure 1) were made to 4% in trichloroacetic acid and the resulting suspension was centrifuged. The CCl₃COOH precipitate (AT, Figure 1) was resuspended in acetic acid and the CCl₃COOH removed by addition of IRA-400 acetate resin (Rohm and Haas, Darmstadt, Germany). When the solution had completely cleared, it was passed through a 1.5 × 20 cm column of IRA-400 and the eluate was lyophilized.

Measured aliquots were removed at successive stages of the extraction (AE, AS, AT; Figure 1) for radioimmunoassay, and a weighed aliquot of the acetone powder starting material (AP, Figure 1) was also retained for assays to assess overall recovery.

A 20-mg aliquot of CCl₃COOH precipitate from the initial extraction (AT-1) was also retained for calibration against the purified hormone, for use as a future radioimmunoassay standard for HPTH.

Gel Filtration. A 2.5 × 130 cm column of Bio-Gel P-100 (Bio-Rad, Riverside, Calif.) was equilibrated with 0.14 M ammonium acetate (pH 4.9) at 4°. The column was presaturated by application of 500 mg of a CCl₃COOH precipitate of bovine parathyroid hormone (BPTH) containing about 50 mg of hormone. The BPTH was eluted with the same buffer and then 2 l. (four column volumes) of buffer was allowed to pass through the column before application of any HPTH.

A flow rate of 20 ml/hr was used in all runs. Optical density of eluate tubes was read at 250 and 280 mµ using the Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif.). Elution of hormone was defined by radioimmunoassay, and the appropriate tubes pooled and lyophilized twice.

Ion-exchange chromatography was carried out using a 0.9 × 10 cm column of carboxymethylcellulose (CMC) (Whatman CM-52; Reeve-Angel, Inc., Clifton, N. J.). Prior to use for the human hormone, the CMC column was presaturated by
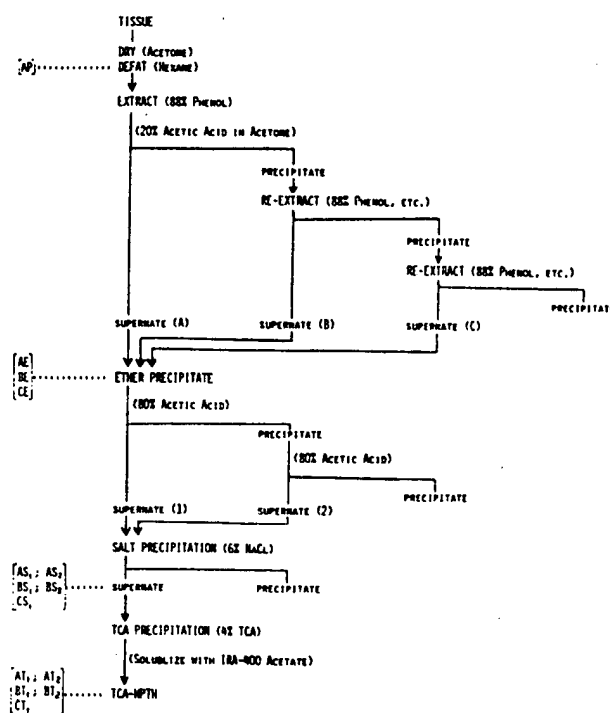
FIGURE 1: Outline of procedure for extraction of parathyroid hormone from pooled human gland tissue. Code designations for various reextracts of the phenol residue and ether powder (described in the text) appear along left margin.

chromatography of 20 mg of partially purified (Sephadex grade) bovine parathyroid hormone. After elution of the BPTH with high ionic strength buffer, the column was exhaustively reequilibrated with the starting buffer, 0.01 M ammonium acetate (pH 4.9, conductivity 0.6 mmho). The lyophilized human hormone pool from P-100 chromatography was applied in the same buffer, and a linear gradient of increasing conductivity established at 4° by means of a Varigrad gradient maker (Buchler Instruments, Fort Lee, N. J.), using 0.2 M ammonium acetate (pH 6.0, conductivity 12.5 mmhos) as second buffer. The most satisfactory linear gradient was achieved by use of two chambers (each 90 ml) of starting buffer and a third chamber (also 90 ml) of second buffer. Following completion of the gradient, 1 M ammonium acetate (pH 6.5, conductivity 30 mmhos) was allowed to run through the column. All conductivity measurements were taken at 20° using the Radiometer (Copenhagen) Model CDM-2e conductivity meter.

For CMC chromatography employing buffers in 8 M urea, the buffer constituents were added to the freshly deionized urea immediately before chromatography, which was carried out at room temperature. Column dimensions and buffer parameters were otherwise the same as described above.

Radioimmunoassays. Recoveries of hormone at the initial stages of extraction were assessed by use of the immunoradiometric assay as described by Addison *et al.* (1971) using ¹²⁵I-labeled antibodies extracted from guinea pig 199, an anti-bovine antiserum (O'Riordan *et al.*, 1972). The reference preparation was a CCl₃COOH precipitate prepared earlier (O'Riordan *et al.*, 1971b), designated HT-67. Radioiodine was purchased from Amersham Ltd. (Amersham, England).

The hormone preparations AE, AS, and AT (Figure 1) were diluted and added directly to the assay. Aliquots of the acetone powder starting material (AP, Figure 1), in amounts of 10–30
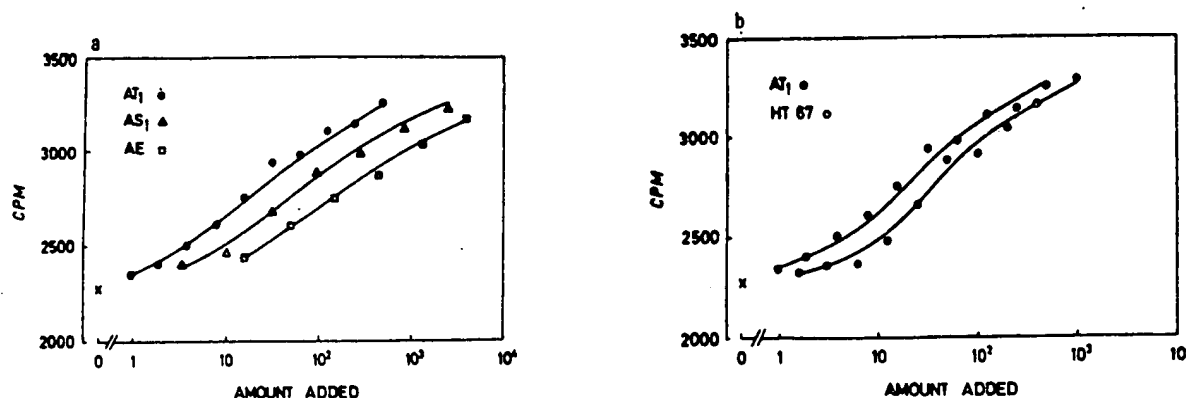
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FIGURE 2: (a) Comparison of response curves for human parathyroid hormone aliquotted from successive stages of initial extraction, in an immunoradiometric assay using ^{125}I-labeled GP-199 antiserum (Addison *et al.*, 1971): AE, ether powder; AS$_1$, salt supernatant; AT$_1$, CCl$_3$-COOH precipitate. Ordinate represents counts of hormone-bound, labeled antibody remaining in supernatant after precipitation of unbound antibody with solid-phase immunadsorbant. Abscissa represents increasing volume aliquots of the respective extracts added into the assay. (b) Comparison of immunoassay response curves for CCl$_3$COOH-HPTH preparation AT-1 from current purification and CCl$_3$COOH-HPTH preparation HT-67 previously prepared by O'Riordan *et al.* (1971b). Assay conditions and coordinates are as described for part a.

mg, were extracted either with 20% acetic acid in 8 M urea or with 90% phenol (2 ml, 18 hr, room temperature for either method) prior to immunoassay.

Recovery studies during the steps of purification subsequent to the CCl$_3$COOH stage were carried out using a modification (Segre *et al.*, 1972) of the standard radioimmunoassay as developed for bovine parathyroid hormone by Berson *et al.* (1963). Guinea pig 1 antiserum, also an anti-bovine preparation (O'Riordan *et al.*, 1969) was used at a dilution of 1:300,000. The same CCl$_3$COOH-HPTH standard (HT-67) was used as a reference in these assays. ^{125}I for labeling of purified BPTH tracer was obtained from New England Nuclear (Wilmington, Mass.).

Scanning of the column profiles for location of eluted hormone was performed using a shortened assay procedure. Guinea pig 1 antiserum was used at a dilution of 1:50,000, enabling incubations to be carried out overnight instead of the usual 3 days. In these assays 20-μl aliquots from selected tubes were assayed in three successive 50-fold dilutions.

Edman Degradations. Chemical purity of the hormone following P-100 and CMC chromatography was evaluated using the phenyl isothiocyanate procedure of Edman (1960). Most degradations were carried out by the automated procedure (Edman and Begg, 1967); aliquots containing 3-5 nmol of peptide were subjected to several steps of degradation in the Beckman Sequencer, Model 201 (Beckman Instruments, Palo Alto, Calif.) using [^{35}S]phenyl isothiocyanate (Amersham-Searle, Arlington Heights, Ill.) to improve sensitivity of detection (Jacobs *et al.*, 1973). Manual degradations were

TABLE I: Recovery of Immunoassayable Hormone from Extraction of 520 g of Pooled Human Parathyroid Gland Tissue.

Preparation	Dry Weight	Hormone Content
Acetone powder (AP)	96 g	85 mg
CCl$_3$COOH precipitates		
AT-1	394 mg	16.0 mg
AT-2	160	2.0
BT-1	218	4.5
BT-2	38	0.3
CT-1	124	1.0

carried out using previously described techniques (Niall and Potts, 1970). Phenylthiohydantoins were identified by thin-layer chromatography accompanied by autoradiography (Edman and Begg, 1967; Jacobs *et al.*, 1973) and by gas-liquid chromatography (Pisano and Bronzert, 1969).

Amino Acid Analyses. Amino acid analysis was carried out both to provide compositional information and to measure protein content of the purified preparations for use in standardizing the radioimmunoassays and biological assays. All hydrolyses were performed using 5.7 N HCl containing 1:2000 (v/v) mercaptoethanol (Keutmann and Potts, 1969). Analyses were carried out using the Beckman Model 121 automatic amino acid analyzer. Amino acids were normalized from mole fractions into moles per mole by best fit based upon recovery of all stable residues.

Bioassays. The *in vitro* potency of the CMC-purified human hormone was assessed using the rat renal-cortical adenylyl cyclase assay system as described by Marcus and Aurbach (1969). [^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear and Schwarz/Mann, respectively. Medical Research Council (MRC) preparation 72/286 (National Institute for Medical Research, Mill Hill, London, England), a highly purified bovine hormone preparation, was used as reference standard.

Results

Preparation of CCl$_3$COOH-HPTH. Following drying and defatting, 96 g of acetone powder was obtained from 520 g of pooled tissue. The initial extraction of this preparation yielded 394 mg of CCl$_3$COOH precipitate (AT-1, Table I) containing 16 mg of hormone by radioimmunoassay.

Immunoassay of the acetone powder itself after small-scale extraction by either urea-acetic acid or phenol showed, however, an immunoreactive hormone content of 80-90 mg (AP, Table I).

Therefore, it was felt that additional quantities of hormone might be obtained by a series of reextractions of the residues from the initial extraction procedure. The tissue residue from the original phenol step was thus extracted and processed a second and third time (Figure 1), yielding an additional 5.5 mg of immunoreactive hormone (BT-1, CT-1; Table I). In addition to these reextractions of the phenol residue, the ether-powder residues from the first two extractions (AE, BE;

FIGURE 3: Elution profile obtained by passage of 273 mg of CCl₃-COOH–HPTH (AT-1) through a 140 × 2.5 cm column of Bio-Gel P-100 using 0.14 M ammonium acetate buffer (pH 4.9). Fraction size was 2.2 ml. Parathyroid hormone, detected by radioimmuno-assay, eluted between tubes 118 and 140.
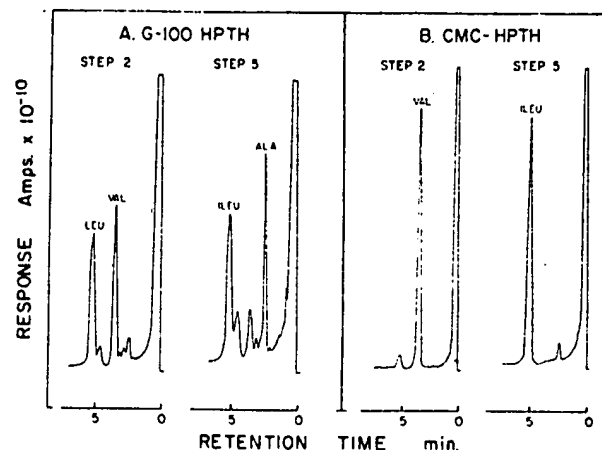

FIGURE 4: Gas–liquid chromatography of phenylthiohydantoins from selected steps of automated Edman degradation of human parathyroid hormone at successive stages of column purification. Valine and isoleucine was derived from the HPTH; the leucine and alanine represented a nonhormonal contaminant present in the P-100 preparation (A) which was eliminated by CMC chromatography (B). The identifications were carried out using a DC-560 column at 180°; isoleucine was confirmed by repeat injection onto an AN-600 column.

Figure 1) were also carried through repeat salt treatment and CCl₃COOH precipitations. This resulted in a further, though modest, enhancement of recovery (AT-2, BT-2; Table I). Overall, 24 mg of immunoassayable parathyroid hormone was recovered at the CCl₃COOH stage; hormone content of the CCl₃COOH precipitates ranged from 4 (AT-1) to 1.5 % (BT-2).

The slope of the displacement curves for the various preparations at the successive steps of extraction were identical with one another (Figure 2a) and with the HPTH standard (HT-67) prepared from the earlier extracts of O'Riordan et al. (1971b) (Figure 2b). Nevertheless, the CCl₃COOH preparations from the various extracts were kept separate as far as was practical through the subsequent stages of purification.

Column Purification. A series of column purifications was performed using successive steps of gel filtration and ion-exchange chromatography. For simplicity the main purification procedure (CCl₃COOH preparation AT-1) is described in detail, and this is followed by consideration of the pertinent features of the purification of the CCl₃COOH preparations derived from the various reextractions.

Purification of HPTH from CCl₃COOH Preparation AT-1. Figure 3 shows the elution profile obtained from P-100 gel filtration of 273 mg of CCl₃COOH preparation AT-1. The peak of immunological activity corresponded to a K_d of 0.3, an elution position similar to that observed for bovine parathyroid hormone using the same type of column, and somewhat earlier than that for BPTH using Sephadex G-100 (Keutmann et al., 1971). An aliquot of the pooled peptide from this region (tubes 113–142) was analyzed by automated Edman degradation. Two predominant amino acids were found at each cycle, in essentially equivalent yields (Figure 4A): step 1, Ser, Val; step 2, Val, Leu; step 3, Ser, His; step 4, Glu, Pro; step 5, Ile, Ala.

The pooled P-100 preparation was subjected to ion-exchange chromatography on carboxymethylcellulose; the elution profile is shown in Figure 5. All of the immunoreactive hormone eluted in a single peak at a conductivity of 7–9 mmhos, a position comparable to that observed for chromatography of BPTH in the same system. Aliquots were taken from across the peak (tubes 124, 128, and 130; Figure 5), as well as from selected tubes adjacent to the peak, for automated Edman degradation. The peptide from all tubes within the

peak retained the sequence Ser-Val-Ser-Glu-Ile-, while a peptide with the sequence Val-Leu-His-Pro-Ala- was found in the eluate fractions following the peak of immunoreactive hormone. This peptide, devoid of immunoreactivity, was felt to represent a non-hormonal contaminant which was responsible for the double sequence seen in Edman degradation of the earlier P-100 pool.

The hormone fractions were pooled and lyophilized. Purity of the peptide (CMC-HPTH) in this pool was found to be 95% based on multiple steps of Edman degradation (Figure 4B). Identical profiles and results were obtained from P-100 and CMC purification of a second lot (107 mg) of CCl₃COOH preparation AT-1.

Purification of HPTH from Reextract CCl₃COOH Precipitates. A third and fourth Bio-Gel P-100 column run was carried out with the reextract CCl₃COOH precipitates, and the elution profiles were closely similar to those described for the preparation AT-1. However, the purity of the hormone in the respective pooled eluates, again assessed by automated Edman degradation, was found to be lower. In the third P-100 pool, derived principally from preparations BT-1 and
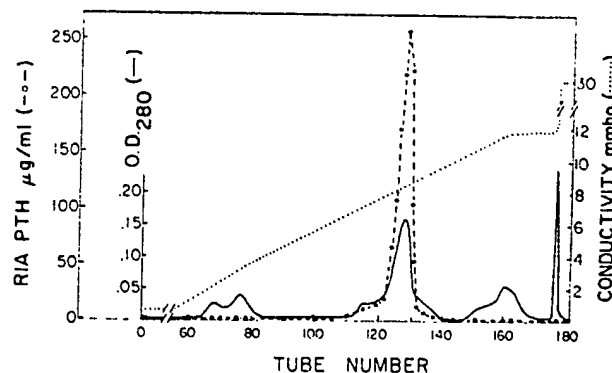
FIGURE 5: Elution profile from chromatography of a P-100 pool of HPTH (Figure 3) on a 10 × 0.9 cm column of carboxymethyl-cellulose using an ammonium acetate buffer gradient as described in text. Fraction size was 1.8 ml. Hormone eluted between tubes 121 and 131.

TABLE II: *In Vitro* Renal Adenylyl Cyclase Activity of Purified Parathyroid Hormone from Various Species.

Hormone	Act. (MRC Units/mg)[a]
Bovine I	3000 (2500–4000)
Porcine	1000 (850–1250)
Human	350 (275–425)

[a] Activity expressed as mean potency with 95% confidence limits.

BT-2, HPTH represented about 25% of the total peptide while in the fourth pool, comprised principally of preparation CT-1, the per cent HPTH was even lower.

CMC chromatography on a pilot scale of material from one of these reextract pools showed that, while the pattern of eluted immunoreactivity remained the same as that for extract AT-1 (Figure 5), the purity of pooled hormone was, as with the pools from gel filtration, significantly lower. In particular, the principal non-hormonal peptide (Val-Leu-His-Pro-Ala-) was found to overlap the later fractions of the immunoreactive hormone peak.

Therefore, the further purification of the reextract P-100 preparations was modified to include a step of CMC chromatography in 8 M urea. Several additional peaks of optical density were found across the profile, but the immunoreactive hormone again eluted in a single peak, at a conductivity of 4–5 mmhos. The eluate pool from this region was diluted with distilled water (to lower the ionic strength) and reapplied to CMC in order to remove the urea. When a gradient was developed using urea-free buffer, the hormone eluted in a profile again resembling that shown in Figure 5. The hormone in this product was 85–90% pure by end-group analysis. A final passage through CMC, using the same urea-free buffer system, increased the purity to a level comparable to that of the CMC–HPTH from the original AT-1 preparation.

Recovery Estimates. In the purification of the initial CCl₃-COOH preparation AT-1, recovery of immunoassayable hormone was found to be similar at each step: 62% from Bio-Gel P-100 and 56% from CMC. Recoveries from P-100 gel filtration of the two reextract CCl₃COOH preparations were somewhat lower: 41 and 45%, respectively. Per cent recovery of these preparations from CMC chromatography was closely similar to the AT-1 material. It was found that use of urea-CMC chromatography was not accompanied by any significant enhancement in recovery. The total yield of highly purified CMC–HPTH, suitable for structural studies, from purification of all the CCl₃COOH preparations was 3.2 mg as calculated from the amino acid analyses.

Biological Assays. Three preparations of CMC–HPTH, quantitated by amino acid analysis, were tested in the renal-cortical adenylyl cyclase system. The assays were found to be statistically homogeneous with a mean potency of 350 MRC units/mg. The relative potencies of human, bovine, and porcine parathyroid hormone in this assay system are compared in Table II.

Amino Acid Composition. The compositional analysis of CMC–HPTH, based on duplicate hydrolysis for 24 and 72 hr, is outlined in Table III. The compositions of BPTH (Keutmann *et al.*, 1971) and PPTH (Woodhead *et al.*, 1971) are also shown for comparison. Results of additional 24-hr hydrolyses of CMC–HPTH, carried out with other preparations for calibration of peptide weight, indicated that all the CMC

products (whether derived from AT-1 or from the various re-extracts) had the same amino acid compositions.

Discussion

Availability of parathyroid tissue starting material has been the limiting factor in efforts to purify human parathyroid hormone. It was also evident from the earlier experience of O'Riordan *et al.* (1971b) that additional problems could also be anticipated in successfully carrying out the purification of sufficient hormone for structural analysis. In particular, it was found that (a) yields of hormone from column chromatography, particularly Sephadex gel filtration, were low and (b) purity, by radioimmunoassay, of HPTH after CCl₃COOH precipitation and gel filtration was considerably lower than that found at the corresponding stages for bovine hormone.

Accordingly, in the current work modifications were made in the later stages of the purification procedure in an effort to offset these problems. Gel filtration was carried out on polyacrylamide instead of polydextran, since in our experience recoveries from the polyacrylamide are somewhat better, perhaps owing to less adsorption of peptide to the column packing. All columns were presaturated with bovine parathyroid hormone prior to use for the human hormone.

By use of these combined maneuvers, it would appear that considerable improvement in yield, especially at the gel filtration step, was obtained, although recoveries were still lower than those found in the course of purification of larger amounts of bovine hormone (Keutmann *et al.*, 1971). Purity of the hormone after gel filtration, as assessed by end-group analysis, was also greater than the estimate of 10% based on radioimmunoassay, reported previously (O'Riordan *et al.*, 1971b).

Use of the manual Edman end-group procedure (Edman, 1960) in monitoring chemical purity of hormone at successive isolation steps proved to be of great value in our earlier work on a larger scale with BPTH (Keutmann *et al.*, 1971). In the current work, with more limited amounts of material, the automated degradation was effectively employed as a rapid, efficient means of screening aliquots across peaks as well as pooled peptide. Only 3–5 nmol of peptide was required for a degradation of several steps, permitting an accurate computation of the content of hormone *vs.* contaminating peptides. Direct chemical evidence of peptide purity was thereby obtained, eliminating the need to consume material through other types of purity assessment such as electrophoretic or chromatographic procedures. The Edman degradation also was useful in providing assurance that the use of BPTH for presaturation of columns to minimize losses was a safe procedure, since in initial pilot-scale column runs the purified HPTH fractions were found to be free from alanine, the amino-terminal residue of BPTH.

The automated Edman degradations showed that a principal contaminant, devoid of immunoassayable parathyroid hormone activity, had been eliminated by the final step of purification (Figure 4). This contaminant appeared different by amino-terminal sequence from the two contaminating peptides which were encountered in the purification of BPTH (Keutmann *et al.*, 1971). Complete purification of the bovine hormone was accomplished by use of CMC chromatography with buffers containing urea. With the principal P-100 preparations of the human hormone, CMC chromatography in the absence of urea was sufficient to yield a pure product. This may be in part a reflection of the differences in contaminating peptides. Moreover, evidence for isohormonal forms,

TABLE III: Amino Acid Composition of Human Parathyroid Hormone.

| Amino Acid | Residues Found (Mol Fraction) | | Residues Taken[a] | | BPTH I | PPTH |
	24 hr	72 hr	Mol/Mol of Peptide	Mol Integer		
Aspartic acid	0.119	0.123	9.80	10	9	8
Threonine[b]	0.017	0.017	1.40	1	0	0
Serine[b]	0.074	0.075	6.10	6	8	8
Glutamic acid	0.118	0.113	9.70	10	11	11
Proline	0.027	0.032	2.30	2	2	2
Glycine	0.056	0.057	4.65	5	4	5
Alanine	0.090	0.092	7.35	7	7	6
Valine	0.093	0.094	7.60	8	8	9
Methionine	0.022	0.015	1.80	2	2	1
Isoleucine	0.012	0.011	0.95	1	3	3
Leucine	0.119	0.116	9.80	10	8	10
Tyrosine	0.002	0.002	0.15	0	1	0
Phenylalanine	0.021	0.020	1.65	2	2	1
Lysine	0.113	0.112	9.30	9	9	9
Histidine	0.058	0.062	4.75	5	4	5
Arginine	0.059	0.059	4.80	5	5	5

[a] The limited quantities of purified peptide available precluded more detailed studies of amino acid composition based on total enzymatic hydrolysis and acid hydrolysis for 24, 48, 72, and 96 hr, to determine the extent of amidation of aspartic and glutamic acid and content of tryptophan, and to more closely examine the content of certain low-yield residues (such as threonine) found to differ in amount from integral values of moles of residue per mole of peptide. [b] Calculated after extrapolation to zero hydrolysis time.

such as those found after urea–CMC chromatography of BPTH (Keutmann et al., 1971), was not found in any of the eluates of the human hormone.

Ion-exchange chromatography in the presence of urea was, however, a useful step in processing P-100 preparations of lower purity, namely, those originating from the tissue reextractions (Figure 1). Keeping the CCl₃COOH preparations from the initial extraction and from the reextracts separate for subsequent purification is therefore clearly warranted. The final purified products from all of the respective extractions were, however, identical by radioimmunoassay and composition.

While the improved yields from the chromatographic steps were gratifying, assessment of yields of hormone from the earlier extraction stages (Table I) showed that substantial quantities of immunoassayable hormone found in the original acetone powder were not accounted for at the CCl₃COOH stage. Hence, the 394 mg of CCl₃COOH precipitate (AT-1) from the initial extraction contained 16 mg of hormone, compared with 85 mg in the acetone powder starting material. For this reason, the various reextractions of acetone powder residue and ether powder were carried out. These yielded additional amounts of hormone, but the total recovery at the CCl₃COOH stage was still only 30%.

The basis for this apparent loss remains uncertain. The greatest drop in immunoassayable hormone content appeared to occur between the acetone powder and ether powder stages. Little or no loss took place at the salt precipitation stage, but appreciable loss again occurred at the CCl₃COOH step. Relevant to this is the finding that reextraction of the original phenol residue yielded more hormone than did reextraction of the ether powder (Table I).

Conceivably, some of the immunoreactivity in the acetone powder could represent fragments which are separated out along the way, despite the fact that the displacement curves for the hormone from the successive stages of extraction appeared to be parallel.

Prior to their independent sequence analysis of the active amino-terminal region of the molecule, Brewer et al. (1972) purified the human hormone starting with a similar quantity of pooled parathyroid tissue. Their procedure employed chloroform, instead of acetone, for drying and defatting of the tissue, and urea–hydrochloric acid rather than phenol for extraction (Arnaud et al., 1970). Although a detailed description of their purification is as yet unavailable, their overall recovery appears to have been similar to our own.

Substitution of serine for alanine at the amino terminus may contribute to the low activity (350 MRC units/mg) of native HPTH, compared to that of the bovine hormone, when assessed by the in vitro renal adenylyl cyclase assay. Earlier studies had demonstrated that porcine parathyroid hormone (PPTH), which also contains an amino-terminal serine residue, likewise shows a lower activity relative to BPTH in the same assay system (Woodhead et al., 1971).

The compositional data obtained in these studies (Table III) point toward significant structural features to be anticipated in analysis of the carboxyl-terminal region of the molecule. Certain similarities to porcine parathyroid hormone may be discerned, including a similar distribution of basic residues, high content of leucine, and absence of tyrosine. However, several differences from both the porcine and bovine molecules are evident as well.

Although the structure for the amino-terminal region proposed by ourselves (Niall et al., 1974) differs in certain respects from that reported by Brewer et al. (1972), it is clear from the compositional studies that, regardless of how these discrepancies are resolved, several amino acids unique to the composition of HPTH should be found in the sequence of the carboxyl-terminal portion. For example, threonine and an aspartic acid residue remain to be located. Also, since the single

isoleucine has already been located (position 5), substitutions would be expected at the two positions occupied by residue in the carboxyl terminus of BPTH and PPTH. Presence in the carboxyl-terminal region of several sequence positions unique to HPTH could account for the low cross-reactivity observed with HPTH against a number of carboxyl-terminal directed anti-bovine antisera (Segre *et al.*, 1972).

The purification of human parathyroid hormone has not only made available peptide for structural analysis, but also has provided a standard, calibrated against multiple CMC preparations, which should be very suitable for future immunological studies. The correlations of structure, biological activity and immunoreactivity which should now be possible will represent important advances toward defining the significance of the various forms of the hormone found in the human circulation.

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ADDED IN PROOF

Subsequent evaluation of the supernatant fraction from CCl₃COOH precipitation (AT-1, Figure 1) showed that a small quantity of intact hormone (about 10% of that appearing in the precipitate) remained behind after the precipitation. This hormone was retrieved in good yield for further purification by the following procedure. The supernatant was extracted with ether to remove trichloroacetic acid. The content of acetic acid (lowered by the ether extraction) was restored to approximately 20%. The hormone was then extracted into 1-butanol, and the butanol removed by rotary evaporation.

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The Amino-Acid Sequence of the Amino-Terminal 37 Residues of Human Parathyroid Hormone

(high-sensitivity automated Edman degradation/radio-iodination/peptide synthesis/immunoreactivity)

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ABSTRACT   The sequence of the amino-terminal 37 residues of human parathyroid hormone has been established. The hormone used in these studies was isolated in highly purified form from parathyroid adenomata and was subjected to automated degradation in a Beckman sequencer. A high-sensitivity sequencing procedure employing ³⁵S-labeled phenylisothiocyanate of high specific activity as the coupling agent was used. The sequence obtained differs from that of bovine parathyroid hormone in three of the first 37 positions, and from that of porcine parathyroid hormone in two positions. A single human-specific residue was found (asparagine 16). The sequence obtained differs at three positions (22, 28, and 30) from the structure for human parathyroid hormone reported recently by Brewer et al. [(1972) *Proc. Nat. Acad. Sci. USA* 69, 3585–3588] and synthesized by Andreatta et al. [(1973) *Helv. Chim. Acta*, 56, 470–473] We have carefully reviewed our data, reported here in detail, on the sequence positions in dispute. We must conclude, on the basis of all available data, that the structure that we propose is the correct structure. The objective resolution of these discrepancies in structural analysis through further chemical and immunochemical studies is important, since synthesis of human parathyroid hormone, in which there is widespread interest for physiological and clinical studies, must be based on the correct sequence of the human hormone if the peptide is to be genuinely useful.

Substantial advances have been made in recent years in our knowledge of parathyroid hormones, through studies of primary structure (1–3), structural requirements for biological activity (4, 5), biosynthesis (6–8), and metabolism (9–14). Most of these studies, including the development and application of radioimmunoassays capable of measuring plasma parathyroid hormone levels in man, have depended directly or indirectly upon the use of the bovine and porcine hormones. Purified human parathyroid hormone (HPTH), on the other hand, has been available in microgram quantities, sufficient only for limited studies of its chemical and immonological properties (15).

Recent improvements in extraction and isolation techniques, and the development of high-sensitivity methods for peptide sequence analysis have permitted us to determine the amino-acid sequence of the amino-terminal biologically active portion of HPTH (Fig. 1).

After the submission for publication in abstract form of our findings for the N-terminal 31 residues of HPTH (24), the report of Brewer et al. (29) of their own independent struc-

tural studies on HPTH was published. Marked discrepancies between the two structures, which differ in three of the first 30 residues, have prompted us to reexamine our data for each cycle of the several degradations performed with the phenylisothiocyanate method.

We now report in full the strategy and methods used in our sequence analysis as well as the quantitative aspects of the results and discuss the nature, implications, and possible approaches to resolution of the differences between the findings of Brewer et al. (29) and ourselves concerning the sequence of the amino-terminal portion of human parathyroid hormone.

MATERIALS AND METHODS

The HPTH used in these studies was extracted from 500 g of pooled human adenoma tissue by use of 88% phenol, followed by treatment with 6% NaCl and precipitation with trichloroacetic acid (15, 16). The hormone was further purified by gel filtration on Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) and ion-exchange chromatography on carboxymethyl-cellulose (Whatman CM-52; Reeve Angel Co., Summit, N.J.) (16). Hormone purification was monitored by radioimmunoassay (11).

Automated Edman degradations were performed on the Beckman model 890 sequencer (Beckman Instruments, Palo Alto, Calif.) using the single-coupling, double-cleavage method of Edman and Begg (17), and other procedures recently described (18). Manual Edman degradations were performed as previously described (19). Reagents and solvents were obtained from Beckman Instruments. ³⁵S-Labeled phenylisothiocyanate was obtained from Amersham/Searle (Arlington Heights, Ill.).

The phenylthiohydantoin (PTH) derivatives were identified by thin-layer chromatography (TLC) on silica gel plates (Analtech, Inc., Newark, Del.) (17, 20) and by gas-liquid chromatography (21) using a two-column system (10% DC-560 and 1.5% AN-600). PTH-histidine was identified by the Pauly reaction (22) and PTH-arginine by the phenanthrenequinone reaction (23). Quantitative yields of the PTH-amino-acid derivatives at each cycle of degradation were determined by comparison with known standards on gas-liquid chromatography. The [³⁵S]PTH-amino acids were separated by TLC; the radioactive spots were identified by autoradiography and quantitated in a Packard model 3375 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Mono-iodohistidine (MIH) and di-iodohistidine (DIH) were synthesized by the method of Brunnings (25) using the modifications of Savoie et al. (26). The phenylthiohydantoin

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Abbreviations: HPTH, human parathyroid hormone; PTH, phenylthiohydantoin; TLC, thin-layer chromatography; MIH, mono-iodohistidine; DIH, di-iodohistidine.

TABLE 1. *Automated Edman degradation of native HPTH*

PTH-amino acids			PTH-amino acids		
Cycle	Found	Yield (nmol)	Cycle	Found	Yield (nmol)
1	Ser	80.0	21	Val	23.8
2	Val	130.2	22	Glu†	10.5
				Thr	7.3
3	Ser	85.3	23	Trp	10.1
4	Glu	71.4	24	Leu	18.7
5	Ile	108.6	25	Arg	13.0
6	Glu*	20.7	26	Lys	14.6
	Gln	35.0			
7	Leu	97.5	27	Lys	15.9
8	Met	51.5	28	Leu	11.0
9	His	42.3	29	Glu*	3.1
				Gln	2.8
10	Asp*	30.6	30	Asp	4.5
	Asn	20.1			
11	Leu	97.5	31	Val	9.1
12	Gly	47.5	32	Ser†	0.7
13	Lys	62.1	33	Asp*	2.7
				Asn	‡
14	His	27.6	34	Phe	3.0
15	Leu	55.5	35	Val	3.0
16	Asp*	17.7	36	Ala	1.4
	Asn	18.1			
17	Ser	43.0	37	Leu	2.1
18	Met	34.8	38	—	—
19	Glu	20.2	39	Ala	1.2
20	Arg	16.4	40	—	—

* Partial deamidation during the conversion reaction accounts for the presence of the free acid as well as the amide form at positions 6 and 29 (glutamines) and positions 10, 16, and 33 (asparagines).

† See *text* for discussion.

‡ TLC identification.

derivatives of MIH and DIH were prepared as described by Edman (27). PTH-MIH and PTH-DIH were separated from all other PTH-amino-acid derivatives by TLC in the solvent system *n*-butyl acetate:water:propionic acid:formamide (240:200:30:60). PTH-[^{125}I]MIH and PTH-[^{125}I]DIH were identified by cochromatography with their respective ^{127}I derivatives followed by autoradiography, and quantitated by counting in a Packard model 3001 gamma well spectrometer.

NH$_2$-SER-VAL-SER-GLU-ILE-GLN-LEU-MET-HIS-ASN-LEU-GLY-LYS-HIS-LEU-ASN
(positions 1–16)

SER 17
MET 18
GLU 19
ARG 20
VAL 21

...LEU-ALA-VAL-PHE-ASN-HIS-VAL-ASP-GLN-LEU-LYS-LYS-ARG-LEU-TRP-GLU
(positions 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22)

FIG. 1. The amino-terminal 37 residues of human parathyroid hormone.

Cleavage of the hormone with cyanogen bromide (CNBr) was carried out in 70% formic acid for 12 hr, 20°, with a 100-fold molar excess of CNBr. Digestion with TPCK-trypsin (Worthington Biochemical Corp., Freehold, N.J.) was performed in 0.2 M trimethylamine acetate buffer (pH 9.2) at 37°, for a period of 2 hr using an enzyme-to-substrate ratio of 1/100.

RESULTS

Purified HPTH (140 nmol) was subjected to automated Edman degradation for 40 cycles. The PTH-amino acid derivatives identified at each cycle of this degradation, and their yields, are presented in Table 1. To illustrate repetitive yield these results are also plotted in Fig. 2.

As shown in Table 1, unique amino-acid assignments, and quantitation of the single residue identified, were possible at all but two of the first 37 cycles of this degradation. At cycle 22, evidence was obtained for two residues, threonine and glutamic acid. Since the quantitative recovery of both of these residues can be low, further experiments were performed prior to definitive assignment of position 22. At cycle 32, a rise in PTH-serine above background levels was observed. However, its yield (Table 1) was considerably below that expected, even for the labile phenylthiohydantoin derivative of serine (18). Although histidine is present at this position in porcine and bovine parathyroid hormones, this residue could not be detected either by the Pauly method or by a definite increase in radioactivity associated with [^{35}S]PTH-histidine at this cycle. However, since the overall yield at this stage of the degradation was near the detection limits for histidine by these methods, further experiments were performed prior to assignment of this position.

The presence of methionine at positions 8 and 18 of the native hormone accounted for both methionines found by amino-

FIG. 2. Yields of phenylthiohydantoin-amino acids obtained during automated degradation of native human parathyroid hormone. See Table 1 and *text*.
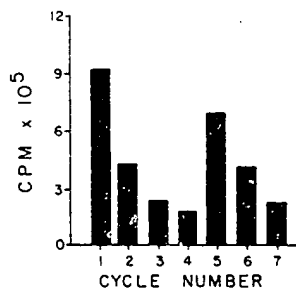
Fig. 3. Release of radioactive (^{125}I-labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during automated degradation of ^{125}I-labeled human parathyroid hormone. The break in the horizontal axis is introduced to simplify the presentation. No significant release of histidine-associated radioactivity was seen in cycles 17-28. [^{125}I]Histidine was found at cycles 9, 14, and 32, indicating the presence of histidine at these positions. See *text*. Numbers on the ordinates are to be multiplied by the indicated factors to obtain the experimental values.

acid analysis (16). This indicated that cleavage of the human hormone with CNBr should result in generation of three principal peptides representing residues 1-8, 9-18, and 19-carboxyl terminus of the native hormone. HPTH (27 nmol) was cleaved with CNBr and the unfractionated peptide mixture subjected to 19 cycles of Edman degradation. The results are presented in Table 2. The expected three end-groups, Ser^1, His^9, and Glu^{19} were identified at cycle one of the degradation. At cycle 4 of the degradation, corresponding to residues 4, 12, and 22 of the intact hormone, only PTH-Glu and PTH-Gly were observed in significant yield. No threonine was detected at this cycle. Therefore, glutamic acid was assigned as residue 22 of native HPTH. The significance of the finding of threonine in the amino-terminal degradation remains uncertain. As can be seen in Table 2, the results of the CNBr mixture analysis also provided complete confirmation of all residue assignments made on the basis of the amino-terminal degradation on intact HPTH.

Since the limited supply of purified HPTH excluded the use of conventional protein chemical methods for reexamination of position 32, an alternative radioactive micro-method was developed to permit detection of histidine residues. Purified HPTH (0.75 μg) was iodinated with ^{125}I by a modification of the Hunter-Greenwood procedure (28). Unlabeled bovine parathyroid hormone was then added as carrier and the mixture was degraded in the sequencer. At each cycle, the radioactivity migrating with PTH-[^{127}I]MIH and PTH-[^{127}I]DIH on TLC was determined. These data (Fig. 3) demonstrate

Fig. 4. Release of radioactive (^{125}I-labeled) phenylthiohydantoin derivatives of mono- and di-iodohistidine during degradation of a tryptic digest of iodinated HPTH. [^{125}I]Histidine was released at cycles 1 and 5. See *text*.

TABLE 2.

Cycle	CNBr1	CNBr2	CNBr3	Yield of PTH Derivative (nmol)
1	Ser^1	His^9	Glu^{19}	Ser 18.3, His*, Gly 27.0
2	Val	Asn	Arg	Val 11.7, Asn 4.3, Arg†
3	Ser	Leu	Val	Ser 10.2, Leu 8.5, Val 18.4
4	Glu	Gly	Glu	Glu 4.5, Gly 3.1
5	Ile	Lys	Trp	Ile 10.2, Lys‡, Trp 5S
6	Gln	His	Leu	Gln 4.9, His*, Leu 10.5
7	Leu	Leu	Arg	Leu 9.8, Arg†
8	Met§	Asn	Lys	Met 3.4, Asn‡, Lys‡
9	His	Ser	Lys	His*, Ser 4.2, Lys‡
10	Asn	—	Leu	Asn‡, Leu 7.5
11	Leu	—	Gln	Leu 1.4, Gln 4.6
12	Gly	—	Asp	Gly 0.9, Asp 2.3
13	—	—	Val	Val 4.2
14	—	—	—¶	—
15	—	—	Asn	Asn‡
16	—	—	Phe	Phe 2.6
17	—	—	Val	Val 4.5
18	—	—	Ala	Ala 2.0
19	—	—	Leu	Leu 2.0

* Identification by Pauly reaction.

† Identification by phenanthrenequinone reaction.

‡ Identification by thin-layer chromatography.

§ Presence of methionine at cycle 8 with the following four residues obtained at cycles 9–12 indicates that cleavage of the Met^8-His^9 bond by cyanogen bromide was incomplete. Residues from this sequence, presumably representing the 1-18 peptide fragment, could not be detected subsequent to cycle 12.

¶ Histidine, subsequently found to occupy position 32 (see *text*) was not detected at the expected cycle (number 14) of this degradation.

the presence of histidine at cycle 32, and confirm the histidine at cycles 9 and 14.

To confirm these results further, and in particular to examine the differences between Brewer *et al.* (29) and ourselves concerning the nature of residue 28, iodinated HPTH was digested with trypsin and then subjected to Edman degradation for seven cycles. The PTH derivatives of [^{125}I]MIH and [^{125}I]DIH were found at cycles 1 and 5 of the degradation Fig. 4). The histidine at cycle 1 further confirmed the Lys^{13}–His^{14} sequence already determined. The finding of histidine at cycle 5 would be predicted on the basis of tryptic cleavage carboxyl to Lys^{27}, and therefore both supports the assignment of His^{32} and argues against the report of Brewer *et al.* (29) that residue 28 is lysine.

DISCUSSION

The amino-terminal sequence we propose for HPTH differs from that of both the bovine and the porcine hormones. HPTH differs from bovine parathyroid hormone (Fig. 5) at positions 1, 7, and 16. The porcine and human hormones differ at positions 16 and 18. Asn^{16} is the only unique residue found in the active region of human parathyroid hormone.

Unexpectedly, our structure differs at three positions from that recently proposed by Brewer *et al.* (29) for the amino-terminal 34 residues of HPTH. They report residue 22 to be glutamine, residue 28 to be lysine, and residue 30 to be leucine. If correct, all these changed residues would be unique to the human hormone. In contrast, we find residue 22 to be glutamic

FIG. 5. Comparison of the amino-terminal sequences of porcine, bovine, and human parathyroid hormones. The central continuous sequence is that of the human hormone (residues 1, 7, and 16). Residues differing in the bovine hormone are *stippled*; those differing in porcine parathyroid hormone (16 and 18) are *hatched*.

acid, residue 28 to be leucine, and residue 30 to be aspartic acid. The residues we have identified are identical with those at the corresponding positions in both the porcine and bovine hormones.

Since both groups have isolated the hormone from essentially similar sources, i.e., human adenoma tissue pooled from many centers, the possibility that there are two hormonal forms which differ as markedly as those of the two proposed sequences is highly unlikely.

We have carefully reexamined our data from both degradations with particular emphasis on the positions in question. In neither degradation was any glutamine observed at cycles corresponding to residue 22. Glutamine and asparagine can undergo deamidation during degradation. However, in both degradations (Tables 1 and 2) glutamine and asparagine residues were detected at cycles beyond position 22, making it implausible that the glutamic acid detected at position 22 was originally glutamine. At cycles corresponding to residues 28 and 30, leucine and aspartic acid were clearly identified by their predominant yields (Tables 1 and 2). The relative rise in yield of these residues above and subsequent fall to background levels is shown in Fig. 6.

In automated Edman degradation the phenomenon of increasing overlap, which tends to be cumulative from cycle to cycle, has been well documented (17, 18, 30). Edman and Begg have, however, found that use of a double-cleavage program can limit this overlap to relatively low levels (17) even in extended degradations. In our amino-terminal degradation, which employed such a double-cleavage program, overlap rose from 4.5% at cycle 12 to 14.5% at cycle 28. Brewer *et al.* reported quantitative data only at position 12; their data permit the calculation that there was a 32% overlap at this early phase of degradation. The natural increase of this already substantial overlap, particularly in view of their use of a single-cleavage program, would make assignment of repeating residues at later cycles of the degradation, such as the putative Lys^{28} in a sequence Lys^{26}-Lys^{27}-Lys^{28}, particularly hazardous.

If, as proposed by Brewer *et al.* (29), residue 28 is lysine, tryptic digestion of $[^{125}I]HPTH$ would lead to cleavage of the

FIG. 6. Yields of PTH-leucine at cycles 26–30 and of PTH-aspartic acid at cycles 28–32 obtained during automated degradation of native human parathyroid hormone. A sharp rise above background levels is seen at cycle 28 for leucine and at cycle 30 for aspartic acid. See *text*.

hormone carboxyl-terminal to residue 28. Therefore, the PTH derivatives of $[^{125}I]MIH$ and $[^{125}I]DIH$ corresponding to His^{32} would be released at the fourth cycle of degradation, rather than the fifth. Clearly, however, histidine is released only at the first and fifth cycles (Fig. 4).

Ultimate resolution of the differences in the proposed structures must await further studies. However, a comparison of our results and methods with the published results and methods of Brewer *et al.* (29) leads us to conclude that our proposed structure based on a variety of approaches is more likely to be correct.

Tests of the biological and immunological properties of synthetic peptides corresponding to our structure and to the structure proposed by Brewer *et al.* (29) for the amino-terminal portion of human parathyroid hormone may prove helpful in objectively resolving the discrepancies in the structures proposed. The marked differences, which include a change in net charge of three within a sequence of nine residues, might affect biological activity. Even more likely is the possibility that such charge differences will result in clear-cut differences in immunoreactivity when the synthetic peptides based on the two proposed structures are each compared with native human parathyroid hormone in their ability to combine with antisera directed against the amino-terminal region (11).

If the structure of Brewer and associates is, as we believe, incorrect, use of antisera generated against the corresponding synthetic peptide for radioimmunoassay studies could confuse rather than aid attempts to more accurately measure HPTH or to understand the complex pattern of metabolism of parathyroid hormone (11). A preliminary immunoassay study based on the peptide of Brewer *et al.* (29) and Andreatta *et al.* (34) has already been published (35). Clearly it is extremely important to establish whether our sequence or that of Brewer *et al.* (29) represents the native HPTH structure before various laboratories embark on extensive immunological studies using synthetic HPTH peptide.

An amino-terminal tetratriacontapeptide based on the structure proposed here has been synthesized by the solid-phase method (31). Studies of the potency of this peptide as measured *in vitro* by activation of renal-cortical adenylate cyclase indicate that its activity is 1030 units/mg, closely equivalent, on a molar basis, to the potency of 350 units/mg (16) for native human parathyroid hormone in this assay. Assays *in vivo* using the chick hypercalcemia assay (32) indicate a potency of 7000 units/mg, an activity identical to that of the bovine peptide 1-34 (no native human parathyroid hormone was available for assay in this system).

The immunological activity of the synthetic peptide has been examined with several antisera directed against the amino-terminal region of parathyroid hormone. Tests against antiserum 199 (33) and GP-1 (11) indicated that reactivity on a molar basis of our synthetic peptide was identical qualitatively and quantitatively to that of the native human hormone. No details have been reported on the specific biological or immunological activity of the synthetic peptide of Andreatta *et al.* (34), whose structure was based on the structure reported by Brewer *et al.* (29). Comparisons based on detailed biological and immunological tests of the two synthetic peptides in various laboratories should be of considerable interest.

Our present findings carry several implications for the structural and comparative immunochemical studies of human parathyroid hormone. The sequence of HPTH we find is identical with that of either the bovine or porcine hormone at 36 of the first 37 residues; the changes found do not affect net charge and do not greatly alter physicochemical properties. Hence, although some improvements in detection of human parathyroid hormone might result from use of antisera directed against the amino-terminal sequence of the human hormone, the improvements, in our view, might not be large. In fact, the success encountered already in numerous laboratories in detection of the human hormone with immunoassays based on the bovine molecule is consistent with the overall chemical similarity found in the amino-terminal sequences of the three species of parathyroid hormone.

On the other hand, previous immunochemical and analytical evidence (11, 15, 16) indicates that more marked differences in structure between bovine and human hormones are likely to be found in the carboxyl-terminal region. Since a large carboxyl-terminal fragment appears to be the major form of immunoreactive parathyroid hormone in the human circulation (11), antisera that recognize the carboxyl end of the human hormone are most likely to significantly improve immunoassay sensitivity. Further sequence studies on HPTH, followed by synthesis of selected peptides from the carboxyl-terminal two-thirds of the molecule, may well result in antisera considerably more sensitive for detection of human parathyroid hormone.

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Human Parathyroid Hormone: Amino-Acid Sequence of the Amino-Terminal Residues 1–34

(automated Edman degradation/mass spectrometry/calcium metabolism/metabolic bone disease)

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ABSTRACT Human parathyroid hormone has been isolated in highly purified form from human parathyroid adenomas. The primary sequence of the amino-terminal 34 residues of the human hormone was obtained by automated degradation with a Beckman Sequencer. The phenylthiohydantoin amino acids were identified by gas chromatography and mass spectrometry. The first 34 residues of human parathyroid hormone differ from the bovine hormone by six residues, and from the porcine hormone by five residues. The amino-terminal residue is serine, similar to the porcine parathyroid hormone; bovine parathyroid hormone contains an amino-terminal alanine. Human parathyroid hormone contains two methionine residues, similar to the bovine species, whereas porcine parathyroid hormone contains a single methionine residue. Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 16, glutamine at position 22, lysine at position 28, and a leucine at position 30.

During the last few years, a significant core of information has been obtained by several laboratories on the chemistry, biosynthesis, and secretion of parathyroid hormone (PTH). These studies have indicated that this hormone is initially synthesized as a prohormone, proparathyroid hormone (1–4). Proparathyroid hormone contains about 106 amino acids, and has an apparent molecular weight of 12,500 (4). The prohormone is rapidly converted into the storage or glandular form of the hormone, which consists of 84 amino acids and has a molecular weight of 9500. The complete amino-acid sequences of the 84 amino-acid parathyroid hormone from bovine (5, 6) and porcine (7) species have been reported. After appropriate physiological stimuli, the 9500 molecular weight form of the parathyroid hormone is secreted into the circulation (8). Shortly after entering the peripheral circulation, the glandular form of the hormone is cleaved into smaller fragments. Gel filtration of human hyperparathyroid serum by several investigators has revealed a major immunoreactive fragment(s), with a molecular weight of 5000–8000, and several minor components (8–10). Immunochemical heterogeneity of the circulating human parathyroid hormone, presumably due to the different molecular forms of PTH, was initially reported by Berson and Yalow (11), and has been confirmed by others (12, 13). The specific site(s) of cleavage in the 84-amino-acid polypeptide chain of the parathyroid hormone in the general circulation is unknown. A biologi-

cally active peptide fragment of bovine PTH, prepared by dilute acid cleavage, has been reported (14, 15), a result indicating that the intact 84-amino-acid polypeptide is not needed for biological activity. This peptide has been identified as the amino-terminal peptide of the hormone, and is composed of the initial 30 residues of the sequence (15). Synthetic peptides of the first 34 residues of the bovine hormone (16) and the initial 30 residues of the porcine hormone (17) have been prepared and are biologically active, thereby confirming the localization of the biologically active region of the parathyroid hormone to the amino-terminal third of the 84-amino-acid polypeptide chain.

The purpose of this communication is to report the amino-terminal sequence of the first 34 residues of human parathyroid hormone, and to compare the amino-terminal sequence of the human hormone to that of the bovine and porcine species.

MATERIALS AND METHODS

The human parathyroid hormone used in these studies was isolated from parathyroid adenomas obtained from patients undergoing surgery for hyperparathyroidism. Dried, defatted parathyroid tissue was initially extracted with 8 M urea in 0.2 N hydrochloric acid, and fractionated with ether, acetic acid, sodium chloride, and trichloroacetic acid (TCA powder) (18). The TCA powder was further purified by gel filtration, followed by ion-exchange chromatography on CM-sephadex with an ammonium acetate gradient. Isolation of the hormone was monitored by radioimmunoassay and disc-gel electrophoresis. The procedures used in the isolation and characterization of the hormone will be described in detail in a separate report.

Amino-acid analyses were performed on a Beckman–Spinco automatic amino-acid analyzer, model 120B or 121 adapted for high sensitivity or with a Durrum model 500 analyzer. Analytical disc-gel electrophoresis was performed in 8 M urea at pH 4.4 (19). Immunoassays were performed by the procedure of Arnaud et al. (20).

Automated Edman degradations were performed with the Beckman Sequencer, model 890B, in 1 M Quadrol buffer. The phenylthiohydantoin (PTH) amino acids were identified by regeneration to the constituent amino acid by hydrolysis with hydroiodic acid for 20 hr at 130° (21), gas–liquid

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Abbreviation: PTH, parathyroid hormone

FIG. 1.   Disc-gel electrophoresis of purified human (*left*) and bovine (*right*) parathyroid hormones.

chromatography (22, 23), and mass spectrometry (24–26). Chemical ionization mass spectrometry was performed on a Finnigin mass spectrometer equipped with a PDP-8/e Digital computer, and a Complot Plotter. Isobutane was used as the carrier gas, and the source was maintained at 200°. The samples were applied by a direct insertion probe, and the probe was heated from 30° to 250° over a 90-sec period. Electron impact mass spectrometry was performed on an LKB mass spectrometer, model 9000, with a direct insertion probe and an electron energy of 70 eV.

RESULTS

The purified human parathyroid hormone migrated as a single component on disc-gel electrophoresis, with a mobility identical to that of the bovine parathyroid hormone (Fig. 1). Amino-terminal analysis of the purified peptide by the Edman technique revealed serine.

350 nanomoles of the purified hormone were degraded on the Beckman Sequencer by use of a single cleavage of hepto-

FIG. 2.   Amino-acid sequence of the amino-terminal 34 residues of human parathyroid hormone.

fluorobutyric acid at each degradation. The results of the degradation of the first 34 residues of the human parathyroid hormone are shown in Fig. 2. The chemical ionization mass spectra of the phenylthiohydantoin (PTH) amino acids obtained at each of the 34 steps in the sequence are shown in Fig. 3. A "quasimolecular" (QM+) or major fragmentary ion is observed in each spectrum (25). At step 12 in the sequence, a quasimolecular ion for glycine (m/e 192) and leucine (m/e 249) are observed (Fig. 3). Quantitation by the gas chromatography method of glycine (0.28 μM) and leucine (0.09 μM) permits definitive identification of glycine as the twelfth amino acid in the sequence, with the leucine resulting from overlap from step 11 (Fig. 2). The ion at m/e 292 and 293 in the mass spectra of step 20 are contaminant ions often observed in variable amounts in the aqueous layer of the Edman reaction. Leucine/isoleucine and lysine/glutamine yield identical masses of m/e 249 and m/e 264, respectively, on chemical ionization mass spectrometry. Lysine, however, can be distinguished from glutamine by the fragmentary ion at m/e 306, as illustrated in the spectra of residues 26, 27, and 28. Lysine/glutamine and leucine/isoleucine were also readily differentiated by gas chromatography on the CFC blend (23) and by electron impact mass spectrometry (24, 25).

These combined results provided a single unique sequence for the first 34 residues of human parathyroid hormone (Fig. 2).

DISCUSSION

The amino-acid sequence of the first 34 residues of human parathyroid hormone is of major importance, since previous studies of the bovine and porcine species have indicated that this is the biologically active region of the native hormone. The first 34 residues of human PTH differ from bovine PTH by six residues, and porcine PTH by five residues (Fig. 4). The amino-terminal 15 residues of human and porcine PTH are identical; however, bovine PTH differs from human and porcine PTH in position 1 and 7, where alanine substitutes for serine and leucine replaces phenylalanine (Fig. 4). In the remaining 16–34 region, human PTH differs from porcine PTH by five residues, and from bovine PTH by four residues (Fig. 4). Human PTH contains two methionine residues— similar to the bovine species—whereas porcine PTH contains a single methionine at position 8 (Fig. 4). The human sequence is unusual in that it contains four consecutive basic residues (arginine residue 25, and lysine residues 26–28). Amino-acid residues in the first 34 that are unique to the human sequence include an asparagine at position 16, glutamine at position 22, lysine at position 28, and a leucine at position 30.

One of the major problems in the clinical assessment of patients with disorders of mineral metabolism has been the difficulties encountered with the radioimmunoassay of human parathyroid hormone. There have been two basic problems with the immunoassay of PTH. The first problem, as discussed above, has been the presence in the peripheral circulation of peptide fragments of the 84 amino-acid polypeptide chain (8–10). Antisera from various laboratories undoubtedly have immunological determinants for different regions of the intact molecule, thus leading to variable and sometimes inconsistent results when applied to the measurement of PTH circulating in human blood (27). In addition, the differentiation by immunoassay of biologically active

FIG. 3. Mass spectra of the phenylthiohydantoin (PTH) amino acids obtained during the automated Edman degradation of the amino-terminal 34 residues of the human parathyroid hormone.

amino-terminal fragments from inactive fragments has so far been impossible. The second difficulty has been the utilization of heterologous assays that use radioactively labeled bovine hormone as the tracer, and antibodies prepared against the bovine or porcine hormone (28–30, 20). The sensitivities of these assays are variable, and depend on the cross reactivity of the particular antiserum with the human hormone. As noted above, the human sequence in only the initial third of the molecule differs from the bovine by six residues and the porcine by five amino acids.

Habener et al. (31) have attempted to circumvent some of these problems with the immunoassay by the development of

amino- and carboxyl-specific antisera. These investigators have used an antibody prepared against the bovine hormone, and have absorbed their antiserum with either the synthetic 1–34 bovine fragment, or a 53–84 fragment prepared by chemical cleavage of the native bovine hormone. The amino-terminal specific antiserum was further characterized by displacement with synthetic bovine fragments, and the recognition site of this absorbed antiserum was shown to be directed toward residues 14–19 in the bovine sequence. Using this approach, they have concluded that the major fragment in the human circulation is carboxyl-terminal, and biologically inactive. They were, however, unable to identify the amino-

HUMAN PARATHYROID HORMONE
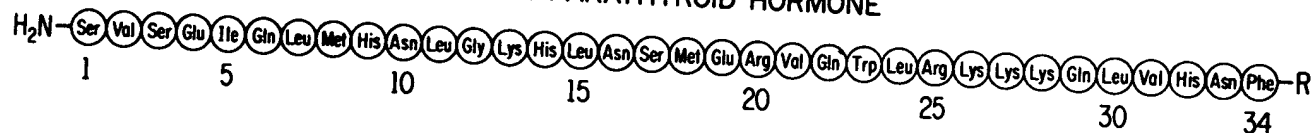
BOVINE PARATHYROID HORMONE
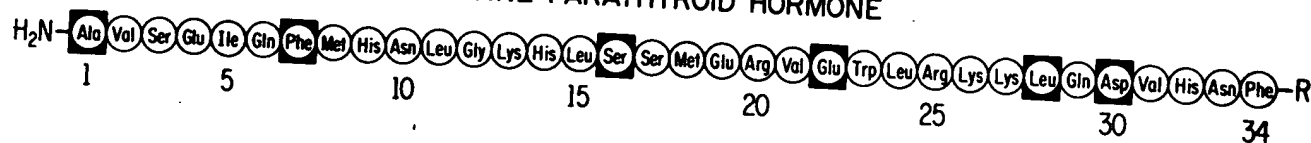
PORCINE PARATHYROID HORMONE
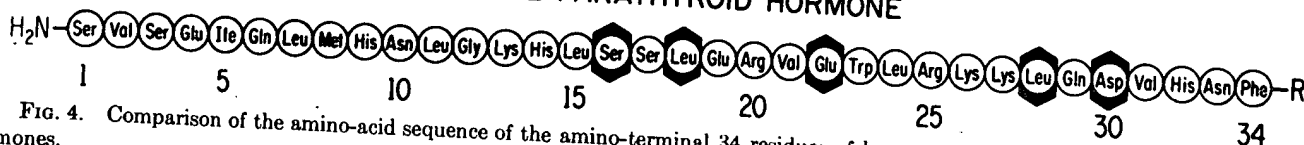
FIG. 4. Comparison of the amino-acid sequence of the amino-terminal 34 residues of human-, bovine-, and porcine-parathyroid hormones.

terminal fragment in the circulation of human subjects. This may be due either to rapid clearance of the amino-terminal fragment from the circulation, or to poor cross-reactivity of the amino-terminal specific bovine antiserum with the amino-terminal region of the human hormone. It is of interest that the human sequence differs in the 14–19 region from the bovine hormone by the substitution at residue 16 of an asparagine for a serine residue (Fig. 4). The significance of this substitution in the human hormone to the results obtained by Habener *et al.* with their amino-terminal specific bovine antiserum is unknown. Canterbury and Reiss have reported results on the nature of the circulating fragment of the parathyroid hormone that are in contrast to those reported by Habener *et al.* Using an antiserum prepared against bovine parathyroid hormone, these investigators have identified three different immunochemical forms of the parathyroid hormone in the peripheral circulation of hyperparathyroid patients (32). The molecular weights of these three components, as determined by gel filtration, were 9500 (presumably glandular PTH), 7000–7500, and 4500–5000. Recently, these investigators have directly assessed the biological activity of these three fragments in a renal adenylate cyclase system (33). Both the 9500 and the 4500–5000 fragment stimulated the adenylate cyclase system, whereas the 7000–7500 component was inactive. These results are consistent with the presence of an amino-terminal biologically active fragment of PTH of about one-half the size of the glandular hormone in human hyperparathyroid serum.

The determination of the amino-terminal sequence of the human parathyroid hormone will now permit the synthesis of peptides based on the human sequence for both clinical and investigative use. Synthetic fragments, as well as chemical analogues, will permit more definitive studies to be performed on the chemistry of the human hormone, including the specific residues and the minimum length of the polypeptide chain that is required for biological activity. In addition, these synthetic fragments will enable investigators to characterize the heterologous antisera currently in use in the immunoassay, and to develop specific antisera directed toward the amino-terminal region of the human hormone. Antisera based on the human sequence will enable more detailed studies to be performed on the nature of the circulating hormone in man, and its role in calcium homeostasis and metabolic bone disease.

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2

DNA Is the
Primary Genetic Material

The realization that genes determine the structure of proteins was a very important milestone in the development of genetics, but it did not have any immediate consequences. As long as the molecular structure of the gene was unknown, there was no way to think constructively about gene–protein relations. In fact, as recently as 1950 there was no general agreement on which class of molecules genes belonged to. Nevertheless, the best guess was that the gene was the deoxyribonucleic acid, a still poorly understood polymeric macromolecule that was just starting to be called by its abbreviation DNA.

DNA Is Sited Exclusively
on Chromosomes

For many years it was hoped that as microscopes improved, it might eventually be possible to see genes sitting side by side along chromosomes. But even with the advent in the early 1940s of the first electron microscopes, which had a potential resolution over 100 times greater than that of light microscopes, there were disappointments. The first electron-microscope pictures of chromosomes showed no repeating pattern at the molecular level; this suggested a highly irregular gene structure that would not be simple to interpret. Attempts to purify chromosomes away from other cellular constituents were much more informative, although it was impossible to obtain really pure chromosomes.

Two main chromosomal components were almost invariably found: (1) deoxyribonucleic acid (DNA), and (2) a class of small, positively charged proteins known as the histones; these, being basic, neutralized the acidity of DNA. DNA had been known to be a major constituent of the nucleus (hence the name "nucleic" acid) ever since its discovery in 1869 by the Swiss scientist Frederick Miescher. In the 1920s, with the DNA-specific purple dye developed by the German chemist Robert Feulgen, DNA was found to be sited exclusively on the chromosomes. DNA therefore had the location expected for a genetic material. In contrast, the histones could apparently be ruled out as genetic components because they were absent from many sperm, which contained instead even smaller basic proteins, the protamines. But most biochemists were not inclined to focus attention on DNA. They thought it would not be nearly as specific as the proteins, of which they knew an unlimited number could be constructed by chaining together the 20 amino acids in different orders. So it was widely believed that some minor and not yet well-characterized protein component of the chromosomes might be found to be the true genetic material.

Cells Contain RNA as well as DNA

Already late in the nineteenth century it had been discovered that cells have a second kind of nucleic acid—what we now call ribonucleic acid (RNA). Unlike DNA, which is located exclusively in the nucleus, RNA is found in the cytoplasm as well as in the nucleus. Within the nucleus, RNA is concentrated in a few dense granules (nucleoli) that are attached to chromosomes.

Both DNA and RNA resemble proteins, in that they are constructed from many smaller building blocks linked end to end. However, nucleotides, the building blocks of nucleic acid, are more complex than any amino acid. Each nucleotide

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The Experimentally Controlled
Introduction of DNA
into Yeast Cells

The baker's yeast *Saccharomyces cerevisiae* is one of the most useful eukaryotic organisms for the study of the regulation of gene expression. Because of its small genome (only four times the size of *E. coli*'s) and short generation time (a few hours), yeast can be experimentally manipulated as easily as most prokaryotes. At the same time, it can be used to study some extremely complex phenomena specific to eukaryotes, including chromosome structure, mitotic and meiotic cell division, RNA splicing, and so on. The genetics of yeast has been well worked out, and hundreds of mutations affecting nutritional requirements, mating, cell division, and radiation sensitivity have been isolated and mapped by conventional methods. Moreover, yeast is a particularly attractive organism for the geneticist because it can be maintained in either the haploid or the diploid state. Complementation between genetic markers can be easily tested by mating pairs of haploid strains that each carry one of the markers. The resulting diploid will reveal whether there is complementation. The diploid can then be induced to undergo meiosis to yield four haploid cells, the products of meiotic division. Recessive markers can be followed easily in the haploids, which greatly simplifies analysis of linkage and recombination. With the advent of recombinant DNA technology and the development of techniques to introduce DNA into yeast cells, this organism has recently been used to make significant advances in our understanding of the molecular biology of eukaryotes.

For example, classical yeast genetics suggested the existence of positive and negative regulatory functions acting to control gene expression. The cloning of these regulatory genes is opening the molecular details of their functions to inspection and manipulation. Various structural properties of the yeast genome have been deduced from the behavior of recombinant DNA when it is transformed into yeast. As we shall describe, these investigations have identified particular chromosomal origins of replication, centromeric sequences (attachment points to the mitotic spindle), and functional telomeres (chromosomal ends). In addition, these studies have provided insight into the genetic properties of tandem reiterated sequences, like the ribosomal RNA genes, and dispersed repeated sequences, like the transposable elements.

Further, the availability of cloned genes provided an opportunity to study the structure of the chromatin at particular genes and to investigate the role of chromatin proteins in the regulation of gene expression. Finally, using yeast it is possible to mutagenize cloned genes (Chapter 8, page 109), reintroduce the mutant genes into their proper positions in the genome, and then assess the effects of the mutations on the genes' function. This "reverse genetics" will expand significantly the number of alleles of a given locus that can be obtained, and will increase our knowledge of the way in which the information in the DNA is inherited, regulated, and ultimately expressed.

Yeast Spheroplasts Take Up Externally Added DNA

DNA can be introduced into yeast quite easily. The yeast cellulose cell wall is removed enzymatically to produce "spheroplasts." The spheroplasts

are then exposed to DNA, CaCl$_2$, and a polyalcohol (such as polyethyleneglycol) that makes the membrane permeable and allows entry of the DNA. The spheroplasts are suspended in agar and allowed to regenerate a new cell wall.

Expression of Yeast Genes in *E. coli*

A major breakthrough in the cloning of yeast genes for use in transformation was the discovery that some of them could complement mutations in *E. coli*. Thus, the leu2 gene of yeast, which codes for an enzyme (β-isopropylmalate dehydrogenase) in the leucine biosynthetic pathway, can complement the leuB mutation in *E. coli*. This is probably not because the *E. coli* RNA polymerase recognizes the yeast gene promoter, but rather because the *E. coli* polymerase occasionally transcribes stretches of yeast DNA at random. If one of these transcripts includes a yeast structural gene, the RNA can be translated into a functional enzyme (assuming that the yeast gene does not contain introns). About 30 percent of yeast genes are found to be functional in *E. coli*, and although this can be thought of as a cloning artifact, it is a very fortuitous one. The total DNA of yeast cells can be cloned into plasmids, which are then used to transform *E. coli* cells carrying appropriate mutations. Bacterial cells receiving plasmids carrying the corresponding yeast gene can then be selected. Several yeast genes coding for biosynthetic enzymes in the tryptophan, histidine, arginine, and uracil pathways (for example, trp1, his3, arg8, and ura3) have been directly cloned in this manner. The plasmid DNA can be extracted from the bacteria and then used to transform mutant yeast strains using the spheroplast method just described.

Shuttle Vectors

With the advent of efficient methods for transforming yeast spheroplasts, the cloning of yeast genes by complementation of mutations in *E. coli* as described above has been superseded by direct complementation of mutants in yeast itself. The method also depends upon so-called shuttle vectors, plasmids that contain both bacterial sequences that signal DNA replication in *E. coli* and sequences that signal DNA replication in yeast. After digestion with appropriate restriction en-

zymes, total yeast DNA is inserted into shuttle vectors and propagated in *E. coli*. The mixed population of plasmids is introduced into yeast spheroplasts. Any recombinant plasmid that complements a mutation in the recipient spheroplasts under selective conditions can be identified and reintroduced into *E. coli* and grown in large amounts (Figure 12-1). The important point is that direct complementation in yeast is not limited to genes for metabolic or biosynthetic enzymes. Any gene for which a mutation can be identified can, in principle, be cloned in this way.

Yeast Also Contains a Plasmid

Most strains of yeast contain an autonomously replicating ring of DNA called the 2μ circle
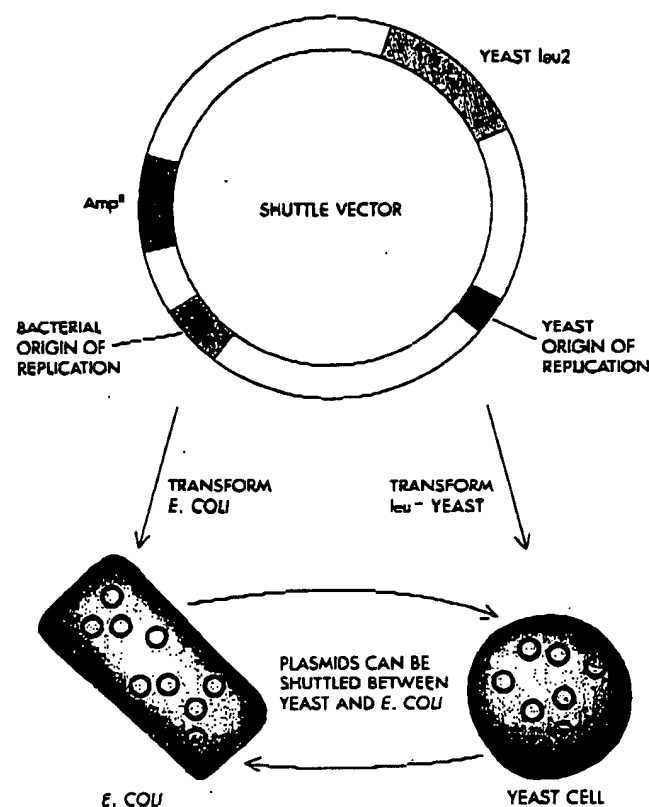
Figure 12-1
Shuttle vectors contain DNA sequences that allow replication in *E. coli* as well as sequences that allow replication in yeast. Such plasmids can thus be shuttled back and forth between the two organisms.

(Figure 12-2). This yeast plasmid, about 6300 bp in length, is present in the nucleoplasm of yeast at about 50 copies per cell. The 2μ DNA is packaged into nucleosomes that have a normal complement of histones. Like most bacterial plasmids, the 2μ circle contains a single origin of replication. In addition, the yeast plasmid itself encodes two so-called "REP" functions (presumably proteins, although they have not been identified) that promote amplification of the 2μ circles when the copy number is low. In this way the stability of the 2μ circle in yeast can be maintained even in the absence of attachment to the mitotic apparatus of the cell. Under normal conditions, the 2μ circle replicates at the same rate as the rest of the genome. When the copy number drops, however, the REP proteins can apparently override the normal coupling of plasmid replication to the cell cycle and initiate multiple rounds of independent replication of the 2μ circle, until the copy number is brought back to 30 to 50 per cell.

Increasing Transformation Efficiency by Addition of Replication Origins

Transforming DNA can be established in yeast either by integration into the chromosome or by autonomous replication as an episome. The presence or absence of ARS (autonomously replicating sequence) elements determines the fate of the introduced DNA. High-efficiency episomal transformation is achieved by including on the circular plasmid a DNA segment that contains an origin of DNA replication (an ARS), which allows replication in the yeast cell independently of the yeast chromosome (Figure 12-2). The presence of an ARS element on a plasmid frequently allows as much as 1 percent of a yeast spheroplast population to be transformed.

Such ARS segments have been isolated from the indigenous yeast plasmid (the 2μ circle) and from randomly cloned segments of the yeast chromosome. They contain at least 60 bp, are AT-rich
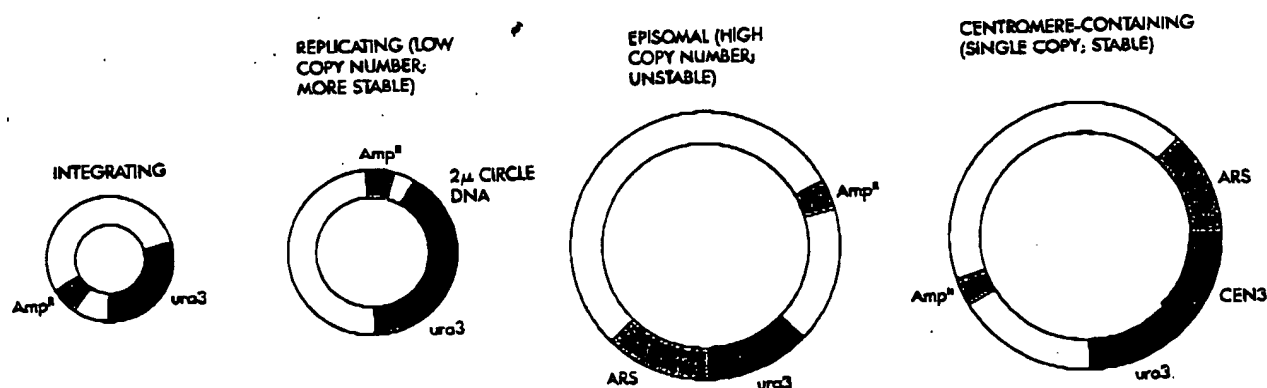
Figure 12-2

Four yeast plasmids. An integrating plasmid contains a yeast selectable marker but no yeast origin of replication. Such plasmids will be stable in yeast only by integration into the yeast chromosome. A replicating plasmid contains a yeast selectable marker and a segment of DNA from the yeast 2μ circle. The 2μ-circle DNA contains the origin of replication and also the "rep" genes, which stably maintain the plasmid extrachromosomally at a relatively low copy number. An episomal plasmid contains a segment of DNA (the ARS sequence) that allows the plasmid to replicate autonomously in yeast cells; however, there is no mechanism to *maintain* such extrachromosomal plasmids at high copy number during mitosis, and these ARS-containing plasmids are unstable. Stability is achieved by adding a CEN sequence, a segment of DNA from one of the yeast centromeres that bind to the spindle apparatus during mitosis. The CEN sequence ensures stable segregation of the extrachromosomal plasmid during mitosis.

(about 80 percent) and have within them the consensus sequence AAA$_T^C$ATAAA. Interestingly, segments of DNA that have been cloned from maize, *Dictyostelium,* and *Drosophila* also function as ARS elements in yeast plasmids. While ARS elements from these various sources function as signals for extrachromosomal replication of DNA introduced into yeast, there is no firm evidence as yet that these sequences function as sites of initiation of *chromosomal* DNA replication in yeast or in any of the other species. So the presence of these sequences in the chromosomes of many diverse species may simply be the result of chance.

Stabilizing Yeast Plasmids with Yeast Centromere DNA

When recombinant bacterial plasmids such as pBR322 containing inserted ARS elements and foreign genes are introduced into yeast cells, the efficiency of transformation is high. But usually the plasmid is lost from the cells as they multiply if the selective pressure is removed. After ten generations only about 5 percent of the cells still have the plasmid. During cell division the plasmids apparently do not segregate regularly and uniformly between the two daughter cells. To overcome this difficulty, DNA segments containing sequences from the centromere (CEN) region of yeast chromosomes can be introduced into the plasmid (Figure 12-2). These sequences ensure the attachment of the chromosomes to the spindle fibers of the mitotic apparatus, and therefore effect the equal segregation of the chromosomes when the cell divides. Plasmids containing these CEN sequences are thus stably maintained by the same mechanism that ensures equal segregation of chromosomes.

CEN sequences were cloned, identified, and isolated by the chromosome-walking procedure. Two genetic markers that had been mapped by classical genetics and so were known to be close to, but on either side of, the centromere of chromosome 3 were selected. Clones from a yeast DNA library with segments of these genes were identified. Further clones overlapping the first pair were selected. The DNAs were then sequenced until the sequence of the complete segment from

one gene to the other and spanning the centromere was obtained. The cloned DNA fragments were introduced individually into shuttle vectors, grown in *E. coli,* and then introduced into yeast. Plasmids carrying one particular segment of DNA were stably maintained in the host yeast cells and were presumed to contain the centromere of chromosome 3. DNA segments with stabilizing activity have also been isolated from chromosomes 4 and 11 by walking from centromere-linked markers on those chromosomes. Sequence comparison of the segments from chromosomes 3 and 4 have revealed several short blocks of sequence homology flanking an AT-rich region 80 to 90 base pairs in length.

In addition to ensuring equal segregation during mitosis, a *bona fide* centromere would be expected to obey the rules of centromeric segregation during meiosis leading to gametes. Plasmids containing long stretches of DNA from the centromeric regions (6 to 10 kb) behave as expected in meiosis. With few exceptions, a single CEN plasmid goes to one or the other pole in the first meiotic division and then segregates to both daughters in the second meiotic division. Smaller subcloned regions of this 6-to-10-kb segment are capable of stabilizing plasmids in mitosis, but they do not direct proper segregation at meiosis. This indicates that the fully functional centromere sequence may be longer than that deduced from experiments with only mitotically dividing cells.

With the knowledge that CEN sequences stabilize plasmids, it is now possible to isolate them by randomly cloning total yeast DNA into ARS-containing plasmids and selecting the recombinant plasmids that are stably maintained following transformation. Usually CEN-containing plasmids are maintained at a low copy number in transformants, at about an average of one plasmid per cell. For studies of gene regulation, it is, of course, desirable to have a system in which the gene dosage can be controlled; this is another advantage of CEN-containing plasmids.

In summary, then, the most efficient plasmid vector for yeast transformation would contain: sequences of *E. coli* plasmid pBR322, an ARS sequence, a CEN sequence, a selectable yeast marker gene (such as leu2), and one or several unique restriction enzyme sites to allow insertion of foreign DNA.

Hairpin Loops at the Ends (Telomeres) of Yeast Chromosomes

Yeast transformation has also been useful for defining the structure of telomeres, the ends of eukaryotic chromosomes. The mechanism by which the ends of linear DNA duplicate is not a trivial problem, because DNA polymerase only synthesizes DNA starting from an RNA primer. The replicated linear DNA chromosomes could thus end up with terminal gaps arising from excision of the RNA primers used to commence synthesis at their 5′ ends. Some linear DNA viruses, like bacteriophage λ, avoid the gap problem by forming circular DNA intermediates. Other viruses having linear DNA molecules, like the phages T7 and T4, have the same sequence at the ends of their DNA molecules, so that their early replicating DNA intermediates can aggregate into long end-to-end concatamers which are later cut into complete genome-length molecules.

Another way out of the gap dilemma is to have the two strands of the linear DNA linked to one another at the terminus; that is, in a hairpin loop. When a replication fork proceeds to the end of such a structure, the end of the hairpin is now the center of symmetry of an inverted repeat, which, as we saw previously, can form a cruciform structure. Such a DNA molecule can then be resolved by nucleolytic cleavages on opposite strands to form two daughter DNA molecules, with a gap or a nick in each (Figure 12-3).

The amplified ribosomal DNA (rDNA) genes of the ciliate *Tetrahymena* were the first telomeric structures to be analyzed in detail. It was found that the ends of these extrachromosomal rDNA molecules are indeed hairpin structures. The rDNA telomeres consist of the repeating DNA unit CCCCAA. The number of these C_4A_2 units was found to vary from 20 to 70 in different rDNA molecules, giving length heterogeneity in the sizes of the restriction enzyme fragments that are generated from the ends of rDNA molecules. These *Tetrahymena* rDNA hairpins were ligated to the ends of a linear plasmid containing the yeast leu2 gene and an ARS sequence. When such a plasmid was introduced into yeast spheroplasts, it replicated and remained a linear DNA molecule. This indicated that *Tetrahymena* telomeres can function in yeast. Furthermore, the essential struc-
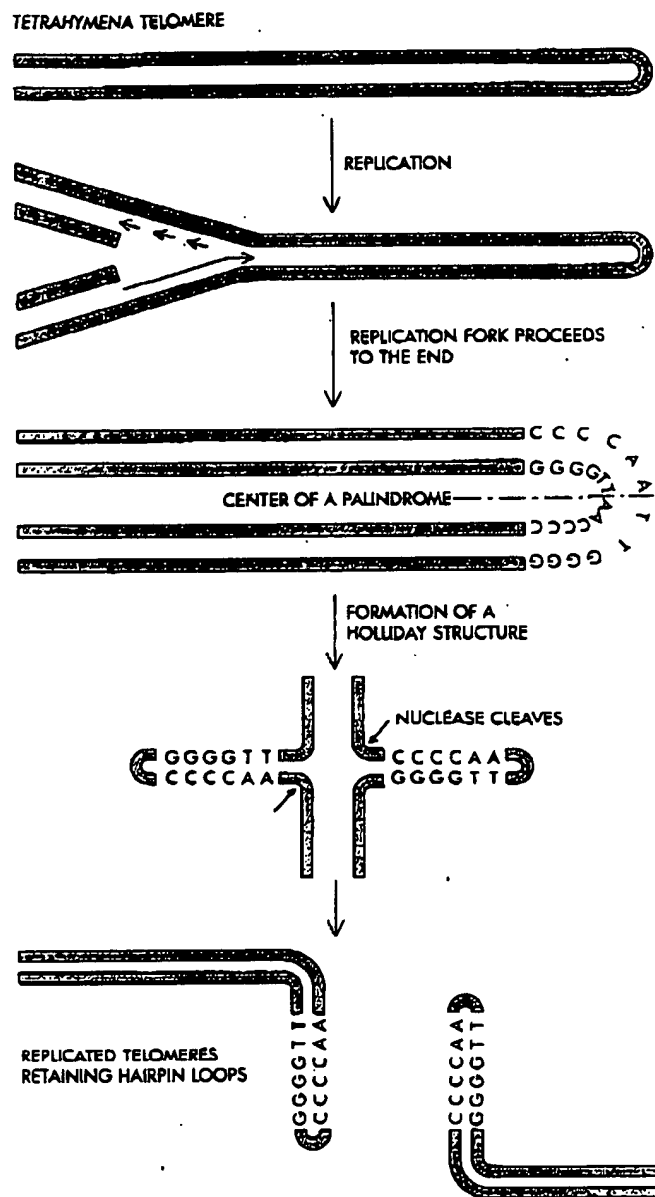
Figure 12-3
A model for telomere replication. The ends of *Tetrahymena* rDNA molecules are hairpin loops consisting of a variable number of CCCCAA repeating units on one strand and GGGGTT units on the other. The replication fork copies out to the end of the DNA molecule. What was the terminus of the molecule is now the center of an inverted repeat structure. The palindromic sequence can form a cruciform or Holliday structure that can be resolved by nucleolytic clips on opposite strands. The result is two replicated daughter molecules with hairpin loops.

ture of the rDNA telomere, that is, its hairpin loop, was maintained (Figure 12-4).

These *Tetrahymena* telomeres were then used to clone chromosomal telomeres from yeast. This was done by constructing a linear plasmid similar to the one just described, but with a *Tetrahymena* telomere at only *one* end. The other ends of the linear molecules were ligated to total yeast DNA that had been cleaved with the restriction enzyme

Figure 12-4
Cloning yeast telomeres. (a) The ends of *Tetrahymena* rDNA (telomeres) containing hairpin loops are ligated to each end of a linear plasmid containing the yeast leu2 gene and an ARS sequence. Such molecules replicate and remain linear when they are introduced into yeast cells. (b) A linear plasmid is constructed as in part (a), but with a *Tetrahymena* telomere at only one end. These molecules are ligated to total yeast DNA that has been cleaved with *Pvu* II. When they are introduced into yeast cells, plasmids that picked up a yeast telomere are able to replicate as linear plasmids.

Pvu II, which generates about 2000 DNA fragments, of which 34 should be telomeres (assuming 17 chromosomes). This ligated DNA was then used to transform yeast cells. Several leu⁺ colonies were found to contain linear plasmids with yeast DNA at one end. Thirty to forty copies of this fragment are present in the total yeast genome, consistent with a model in which every yeast telomere is homologous to the copy that was cloned. Further analysis indicated that the different chromosomal telomeres in yeast are very similar to one another for about 3 or 4 kb from the end; the chromosomal sequences then diverge.

By using smaller and smaller pieces of yeast telomeric DNA, and by introducing specific mutations in this DNA, it should be possible to pinpoint those sequences or structures that are essential for telomere function.

Directed Integration of Cloned DNA into the Yeast Chromosome

DNA transformed into yeast spheroplasts can integrate into chromosomes. Almost all integration occurs by a crossover event between homologous sequences on the incoming DNA molecule and the yeast chromosome. If the DNA is introduced into the yeast cell as a circular molecule, integration turns out to be a very rare event, occurring on the order of 1 in 10⁶ cells, even if the region of homology with the chromosomal sequence is more than 10,000 base pairs. However, if the plasmid is first cut with a restriction enzyme and is then introduced into yeast spheroplasts, it integrates into the chromosome at a site homologous to the cut site about 100 times more frequently than when it is introduced as a circular molecule (Figure 12-5). So it is possible to direct an incoming plasmid to a specific site in the yeast chromosome by cutting it at an appropriate place with a restriction enzyme.

This ability to direct transforming DNA to a specific site in a yeast chromosome can be used to replace a wild-type gene with a mutant one. The procedure, called "allele replacement," allows the study of the effects of a specific gene mutation made *in vitro* on the expression of that gene. Be-
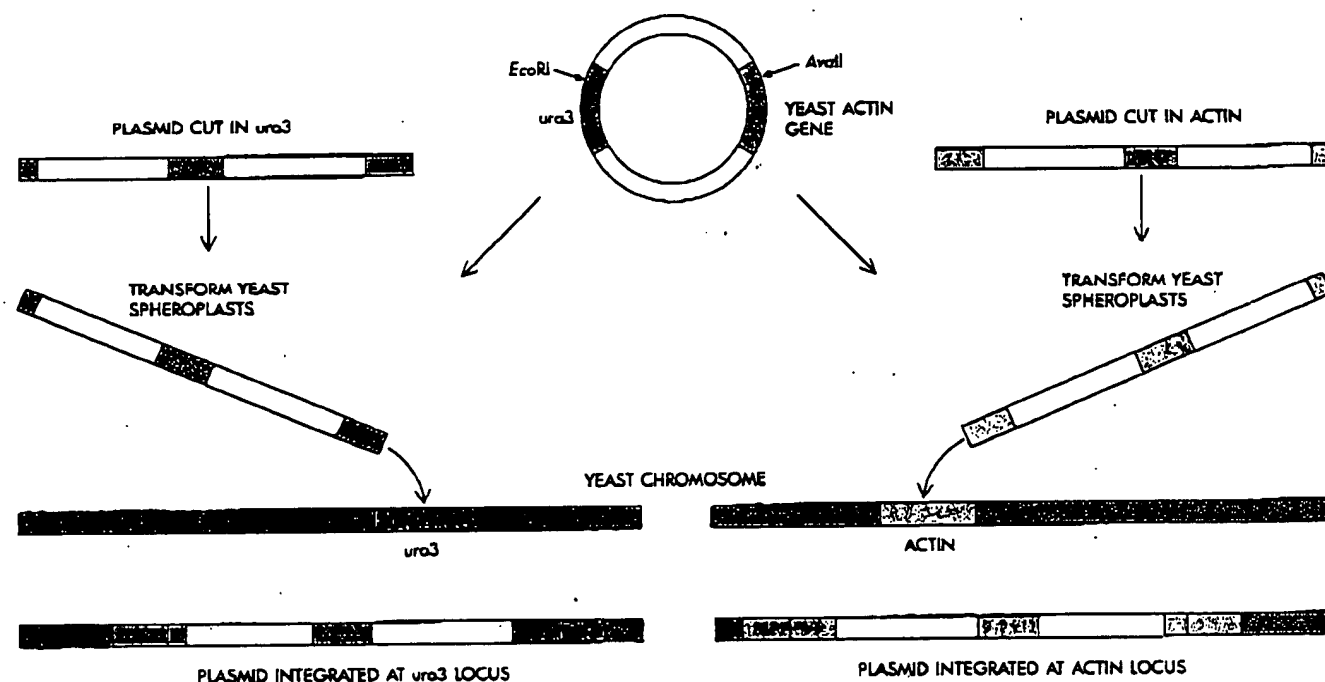
Figure 12-5
Directed integration into the yeast chromosome. An integrating yeast plasmid (that is, one without a yeast origin of replication) can be directed to integrate at a specific site by cleaving the plasmid with a restriction enzyme. For reasons that are unclear, a linear DNA molecule with ends homologous to a sequence in the yeast chromosome is hundreds of times more recombinogenic than a circular molecule. Cleaving a plasmid anywhere in the ura3 gene will cause integration into the ura3 locus in the yeast chromosome, whereas cleavage in the actin gene in the plasmid will ensure integration at the actin locus.

Figure 12-6
A yeast retriever vector. A wild-type yeast gene
is cloned, along with its flanking sequences, into
an integrating yeast plasmid (one without a yeast
origin). The wild-type gene must be flanked by
sites for two different restriction enzymes (here,
Hin dIII and Bam HI). The plasmid also contains
a yeast selectable marker (here, ura3). Such a
clone can be used to retrieve a mutant allele of
the cloned gene from the yeast chromosome:
When a yeast spheroplast is transformed to ura⁺
by one of these plasmids, integration will occur
by a homologous crossover into the yeast
chromosome at the mutant allele. If integration
occurs *within* the gene, the procedure will not
be successful; however, if the crossover occurs in
the flanking sequences, the result will be a
tandem arrangement of the wild-type and mutant
alleles in the chromosome. As depicted here,
subsequent cleavage of such DNA with Hin dIII
and circularization will liberate the wild-type
allele on a plasmid with the Amp^R gene, whereas
cleavage with Bam HI and circularization will re-
sult in the mutant allele being retrieved. (If the
integration had occurred in the 3' flanking se-
quences, then cleavage with Hin dIII and Bam HI
would have given the opposite results.)
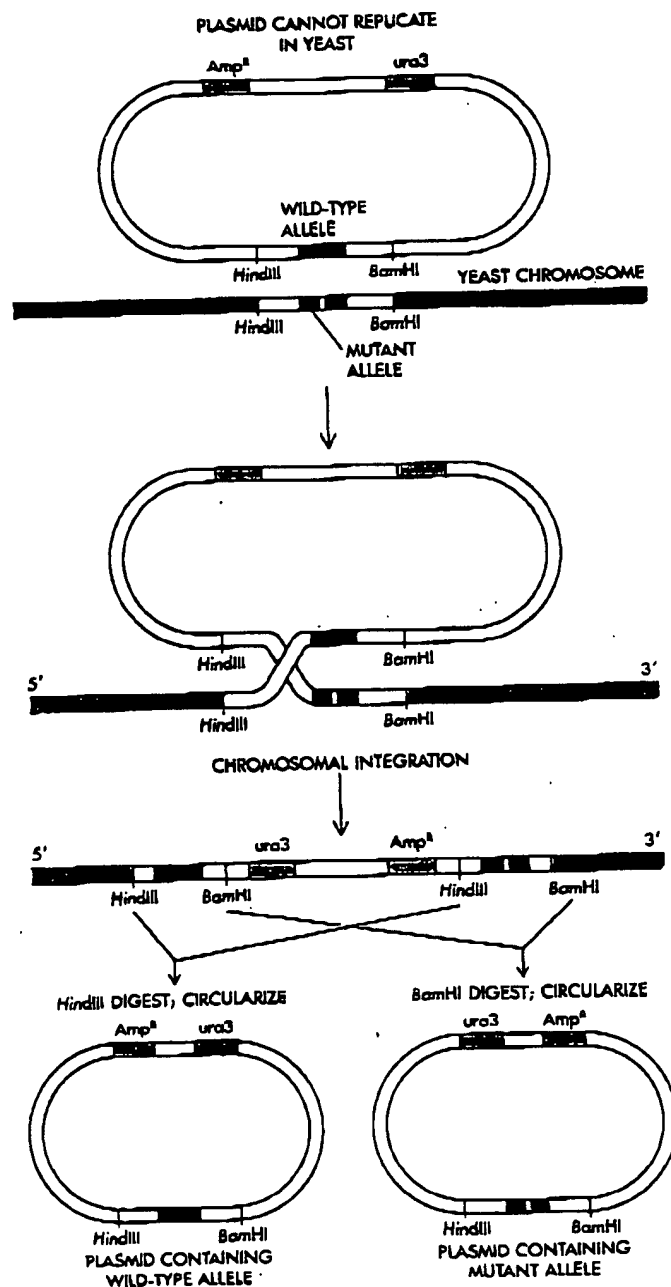
cause the altered gene is sitting in the yeast chro-
mosome at its "correct" site, one can be more
confident that any effects observed are actually due
to the mutation, and not to changes in chromoso-
mal location or changes in copy number. This tech-
nique was used to replace one of the yeast actin
alleles with a mutant actin gene created by site-
specific mutagenic procedures. The result was a
recessive lethal mutation, indicating that func-
tional actin is essential for the survival of yeast.

Retriever Vectors

The fact that integration of a plasmid into the yeast
chromosome occurs by homologous recombina-
tion can be used to isolate naturally occurring mu-
tant alleles of a yeast gene once the wild-type gene
has been cloned. This can be done in either of two
ways.

In one technique, a plasmid containing the
wild-type yeast gene and flanking sequences is con-
structed in such a way that there are two unique
restriction sites, one on each side of the yeast gene

(Figure 12-6). The plasmid also contains the am-
picillin-resistance gene of pBR322 and a yeast se-
lectable marker (such as ura3), but no yeast repli-
cation origin, so that the only way that yeast cells
can be transformed to ura⁺ is by integration of the
plasmid into a chromosome. (Although this is a
low-frequency event with a circular plasmid, it will
occur.) If the recombination of the plasmid with
the yeast chromosome occurs in the flanking se-
quences of the gene, then the chromosome of the
transformed yeast cell now contains two copies of
the gene in tandem: the newly integrated copy
and the endogenous allele (Figure 12-6). Yeast
chromosomal DNA from the transformed cells is
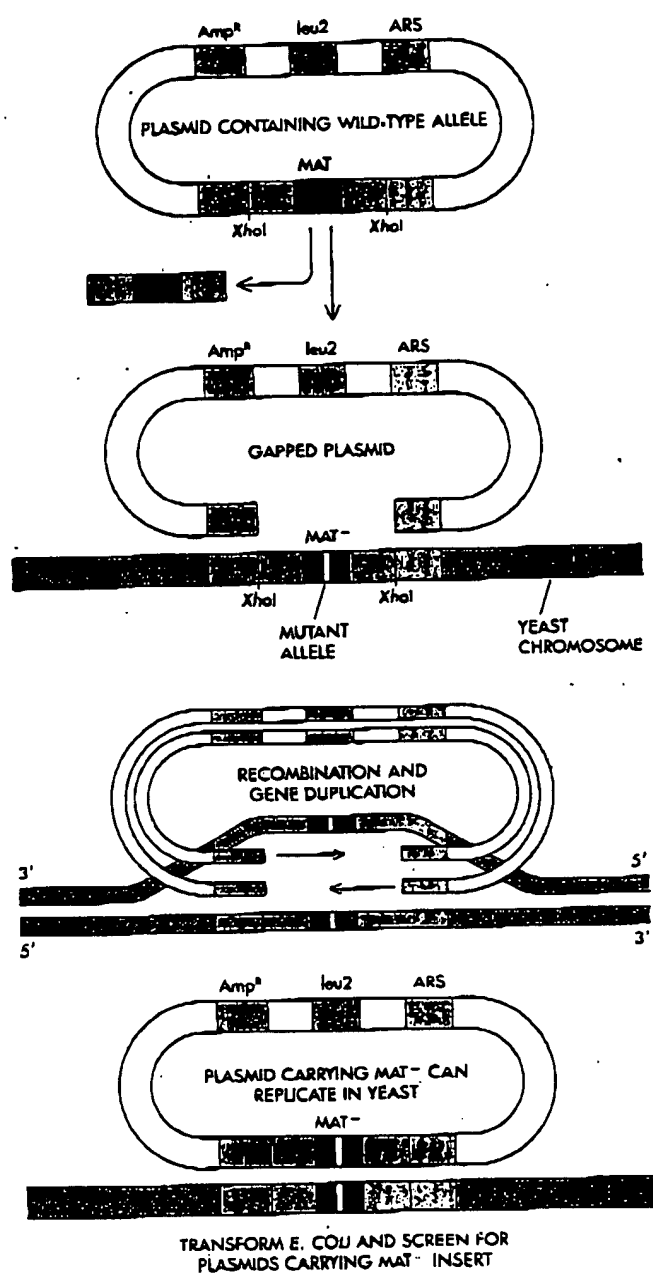
Figure 12-7
Another type of retriever vector. A yeast gene is cloned into a plasmid with a yeast origin of replication. The yeast gene (here, MAT) is then excised by using restriction enzymes, but 5' and 3' flanking sequences remain on the plasmid. When this construction is used to transform yeast cells, the only way it can replicate is to be repaired back into a circle. This can occur if the 5' and 3' flanking sequences of the cloned gene pair up with their homologous sequences in the chromosome. DNA replication will result in the mutant allele from the chromosome being "copied onto" the plasmid, which can then be used to transform *E. coli.*

then cleaved separately with each of the two restriction enzymes whose recognition sites flanked the yeast gene in the plasmid used for transformation. Depending on which side of the resident chromosomal gene the recombination occurred, cleavage with one of the restriction enzymes followed by circularization will result in an ampicillin-resistance plasmid containing the allele that was resident in the chromosome, while cleavage with the other enzyme will result in an ampicillin-resistance plasmid with the original cloned gene (Figure 12-7).

A more efficient method of retrieving mutant alleles from the yeast chromosome is to begin with a plasmid containing a yeast selectable marker, an origin of replication, and a yeast gene with its flanking sequences. Restriction enzymes are used to excise the yeast gene, leaving only the flanking sequences. The result is a plasmid with a large gap in it. When this gapped plasmid is transformed into yeast cells, it must be repaired to a circle in order to replicate; the easiest way for this to occur is for the flanking sequences to recombine with their homologous sequences in the chromosome. These sequences in the chromosome, of course, flank the gene that is to be retrieved. This double recombination event results in the chromosomal copy of the gene being replicated into the gapped plasmid (Figure 12-8). The now circular plasmid containing a copy of the chromosomal allele can proceed to replicate in yeast cells and be isolated.

Gene Organization

By now, several yeast genes have been completely sequenced, including those for the proteins alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, actin, enolase, anthranilate isomerase, mating type (MAT a1 and MAT a2), a-pheromone (sex hormone), and the two forms of cytochrome c. Only one of these genes, that for actin, contains an intron. If this sample of yeast genes coding for proteins is representative of the whole genome, introns must be much rarer in yeast than in higher eukaryotes. In contrast, introns appear to be a regular feature of yeast tRNA genes.

Outside the coding sequences are found sequences of TATAAAA and TATAAA, analogous to the "TATA boxes" of higher eukaryotes. These regions most likely serve as promoter elements.
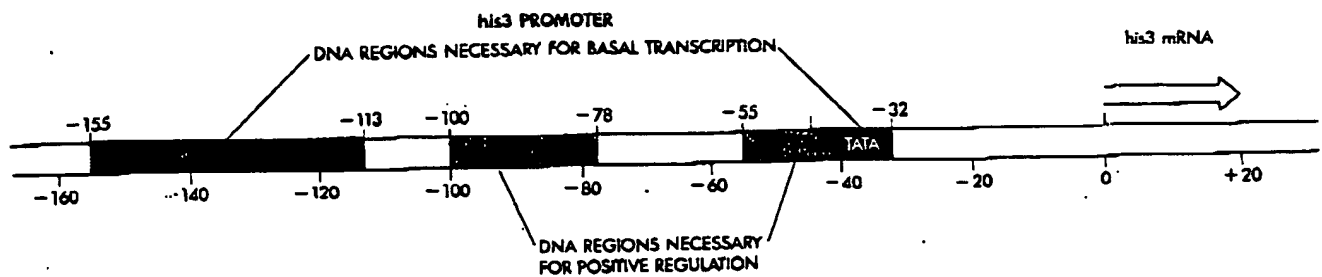
Figure 12-8
DNA sequences responsible for the regulation of the yeast his3 gene. Site-specific mutations were made throughout the promoter region of a cloned his3 gene, and the mutant genes were reintroduced into yeast at the normal his3 locus. It was found that two distinct regions were necessary for basal-level transcription, while two other distinct regions were necessary for the positive regulation of his3 transcription by amino acid starvation.

Much less clear is the nature of the sequences coding for the poly-A-addition sites. Some yeast genes may use AATAAA-like regions, whereas in other genes different sequences seem to play this role.

Regulation of Gene Expression in Yeast

Coordinately controlled genes in yeast are scattered throughout the genome on different chromosomes. This is unlike the situation in bacteria, where coordinately controlled genes are organized into operons (Chapter 4). For example, three of the yeast genes specifying the enzymes of the histidine biosynthetic pathway are on different chromosomes. Analysis of the DNA sequences on the 5' side of these structural genes (the region in which regulatory signals might exist) revealed remarkably little homology; fewer than 14 base pairs of sequence are common to all three regions. The question now is how, given so little homology in supposed regulatory regions, a coordinated regulation of expression is achieved. Clearly, in eukaryotic organisms there seems to be no evolutionary pressure to keep coordinately controlled genes in contiguous blocks, or operons.

Studies of how the individual yeast genes are controlled have revealed that the sequences on the 5' side of many regulated yeast genes, including cytochromes and the histidine biosynthetic enzymes, have *two* components necessary for normal regulated expression. Promoter sequences lie close to the transcription-initiation site and are necessary for basal levels of transcription. In addition, sites for the action of positive regulatory molecules lie more than 100 base pairs further upstream of the site of transcription initiation (Figure 12-8).

Another important result from studies of gene regulation in yeast by recombinant DNA methods is that at least some regulatory functions act at sites very distant from the beginning of the gene being regulated. For example, the HML and HMR silent mating-type genes are not expressed, even though they have completely functional genetic information, including a promoter. To be expressed, copies of the gene must be inserted in the MAT locus (Chapter 11). The two silent genes are kept silent by two sequences of DNA that are many hundreds of base pairs away from the gene. Comparison of the structure of the chromatin at the MAT locus, where expression occurs, with that of the two silent genes reveals significant differences. Apparently, the structure of the chromatin—in other words, the way the DNA is complexed with histones and other proteins—can alter the accessibility of the promoter to RNA polymerase. The same conclusion comes from studies of the yeast transposons, the Ty elements. Insertion of a Ty element hundreds of base pairs upstream from a transcriptional unit of a gene can cause the gene to be constitutively expressed; alternatively, such an insertion can completely shut off the gene's expression. These effects may also be attributed to alterations in the structure of the chromatin.

This correlation of chromatin structure, DNA sequence, and transcription with genetic analysis is greatly increasing our understanding of gene regulatory mechanisms in eukaryotes. The knowledge that such mechanisms exist in yeast, a simple eukaryote, will at the very least make the identification of similar mechanisms in higher eukaryotes much easier.

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13

Genetic Engineering of Plants by Using Crown Gall Plasmids

Over the past 20 years, methods have been developed for redifferentiating whole mature plants from cells growing in culture. These developments have recently been coupled with the discovery that crown gall plasmids of certain soil bacteria naturally integrate into the chromosomes of the plant cells these bacteria infect. As a result, we have begun to exploit an entirely novel method of introducing individual genes into plants—a method that has great potential importance for plant breeding. Many other possible recombinant DNA techniques for plant genetic engineering are being investigated, but the crown gall plasmid system is currently the most advanced.

Conventional Plant Breeding Methodologies

Plant breeding, the production of more useful plants, has by now become a very sophisticated branch of applied Mendelian genetics. The yields of crops like wheat and corn have steadily increased over the last 50 years, and these rates of increase are being maintained. One area of plant breeding that might be amenable to recombinant technology is the transfer of simple traits like disease resistance from one variety or species to another. To introduce a particular desired gene or set of genes by conventional methods, the two lines are sexually crossed to give first-generation hybrids with a genetic constitution derived from both parents. The hybrids are then grown up and repeatedly back-crossed with one parent until a plant with the desired genetic makeup emerges. This plant will have most of the genes of the one parental variety with a few particular desired characteristics from the other; the process is called introgression. Such breeding is necessarily slow and usually spans several years, even when a range of genetic and breeding tricks are used to accelerate it. Furthermore, it is essentially restricted to sexually compatible species that can hybridize with each other; thus, it is limited by the natural species barriers to gene exchange.

Plant Cells in Culture

When a plant is wounded, a patch of soft cells called a callus grows over the wound and, with time, phenolic compounds accumulate in these cells and harden and effectively seal the wound. The hardened callus is the plant's equivalent of scar tissue. If a piece of young, still-soft callus is removed and placed in a culture medium containing salts, sugars, vitamins, amino acids, and the appropriate plant growth hormones, instead of hardening, the cells continue to divide, and give rise to a disorganized mass of relatively undifferentiated cells, a "callus culture." Pieces of tissue taken from inside a plant, or from young seedlings grown under sterile conditions, also give rise to similar cell cultures when the tissue pieces are placed in media containing plant hormones.

Redifferentiation of Whole Plants from Culture Plant Cells

When cultures of plant cells of certain species are exposed to media with appropriate growth hormones, some of the cells can be induced to redif-

The Prostate

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RAPID COMMUNICATION

Measurement of Prostate-Specific Membrane Antigen in the Serum With a New Antibody

G.P. Murphy, W.T. Tino, E.H. Holmes, A.L. Boynton, S.J. Erickson,
V.A. Bowes, R.J. Barren, B.A. Tjoa, S.L. Misrock, H. Ragde,
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ABSTRACT: Work to date has identified prostate-specific membrane antigen (PSMA) as a membrane-bound glycoprotein with high specificity for prostatic epithelial cells. PSMA reacts with the monoclonal antibody 7E11.C5, which is present in serum, seminal fluid, and prostatic epithelial cells, and is increased in its expression in the presence of a hormone refractory state associated with prostatic cancer. This report confirms these results and further documents the presence of the monoclonal antibody 3F5.4G6, which reacts with the extracellular domain of PSMA. This region of PSMA is also an element present in a truncated version of the protein, so-called PSM'. Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species that are reactive with the reciprocal antibody in Western blot analysis. Thus, 3F5.4G6 recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. These two antibodies are well suited for use in a sandwich immunoassay, either one as a capture or detection antibody. Current work on this is underway.

This report also confirms that 7E11.C5 Western blots for PSMA are negative with normal human brain tissue. The monoclonal antibody 9H10 does not react with 3F5.4G6 or with 7E11.C5 in studies conducted herein. Moreover, 3F5.4G6 reacts with PSMA found in the LNCaP cell line, but not DU-145 or PC3, which lack PSMA. © 1996 Wiley-Liss, Inc.

KEY WORDS: prostate-specific membrane antigen (PSMA), prostate cancer, prostate marker

INTRODUCTION

We have previously described an antibody 7E11.C5 that can, by enzyme-linked immunosorbent assay (ELISA) (with another antibody 9H10) or by Western blot, detect in prostate cancer patients a protein called prostate-specific membrane antigen (PSMA) [1–3]. To validate this observation further, we collected sera in a prospective multicenter study. The sera were run on a double-blind basis without knowledge of the clinical state, or the presence or absence of prostate cancer [4]. Furthermore, we compared levels of prostate-specific antigen (PSA) concurrently with PSMA values [4]. The elevated PSMA levels predicted a state of clinical progression or clin-

ical resistance in most cases (>70%). PSMA levels were of better prognostic value than PSA [4]. Despite these observations, there have recently been publications questioning whether PSMA was present in the serum [5,6]. We have completed a series of experiments which validate our original observations, and herein report such.

MATERIALS AND METHODS

Cell Lines and Reagents

P3X63Ag8U.1 (X63), an HPGRT-negative mouse myeloma cell line (CRL 1597 from ATCC, Rockville, MD), was maintained in 90% RPMI-1640: 10% Fetal Clone (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, penicillin/streptomycin, and 1 mM sodium pyruvate. Rabbit antimouse IgM and antimouse IgG were purchased from ICN (Costa Mesa, CA). Peroxidase-labeled goat antimouse IgG and goat antimouse IgM were purchased from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, MD). LN-CaP, a prostate cancer cell line (CRL 1740, from ATCC, Rockville, MD) expressing PSMA, was maintained in 95% RPMI-1640/5% fetal calf serum (FCS).

Preparation of Immunogen and Immunization of Mice

PSMA-derived peptide 716–723 (NH₂-ESKVD-PSK−) was coupled to keyhole limpet hemocyanin (KLH) as a carrier protein using the EDC coupling method of Pierce (Rockford, IL). The peptide–carrier complex was emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO) containing 1 mg/ml muramyl dipeptide (MDP, Pierce) at a final concentration of 250 μg/ml. BALB/c mice were immunized subcutaneously with 100 μl of the emulsified peptide–carrier complex every 2 weeks. Following the third injection, blood was obtained from the mice and their sera were tested for antipeptide antibodies in a peptide-specific radioimmunoassay (RIA). Spleens from donor mice demonstrating an antipeptide titer of 1:1,000 or greater were used in a fusion protocol with X63 myeloma cells.

Fusion Protocol and Initial Screening for Antipeptide Producing Hybridomas

Three days prior to fusion, the donor mouse was immunized intraperitoneally with 50 μg of peptide–carrier complex in saline. The spleen was aseptically removed, and a single cell suspension was prepared in RPMI-1640 medium without serum. The splenocytes were added to X63 myeloma cells at a ratio of 10:1, and the fusion was performed by the method of Galfré and Milstein [7]. Following fusion, the splenocyte–myeloma mixture was resuspended in 80% RPMI-1640/20% Fetal Clone supplemented with aminopterin, to act as a selective medium for hybridoma growth and plated in 200-μl volumes into sterile 96-well microtiter plates.

Ten to 14 days following fusion, 50 μl of cell culture supernatant from the individual wells was removed and tested in an RIA for peptide-specific antibodies. Briefly, the supernatants were added to wells of a Pro-Bind plate (Falcon) coated with PSMA-peptide coupled to bovine serum albumin (BSA) by the EDC method referenced above and blocked with BSA. Following an overnight incubation at 4°C the plates were washed 4 times with PBS-0.1% BSA. Fifty μl of a 1:500 dilution of rabbit antimouse IgM and antimouse IgG was added to each well, and the plates were incubated for 1 hr at room temperature. Following four washes, 50 μl of ¹²⁵I-Protein A (≈10⁵ cpm/well) was added to each well and the plates incubated for an additional hour at room temperature. The plates were washed four times and exposed to X-ray film (Kodak, X-OMAT) overnight and developed. Positive wells showing antipeptide-specific antibodies were identified from the exposed film and the cells from the positive wells were expanded in 90% RPMI-1640/10% Fetal Clone for further testing.

Western Blot Analysis of Primary Hybridomas for anti-PSMA Antibody

Supernatants from the expanded antipeptide-reactive primary hybridomas were tested in a Western blot assay for the presence of anti-PSMA antibodies. Western blot analysis was performed following the protocol of Pelletier and Boynton [8]. Briefly, lysates from LNCaP cells, a prostatic tumor cell line that expresses PSMA, were electrophoresed on an 8.5% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electroblotted onto a PVDF membrane for 1 hr at 90 V. The membranes were blocked overnight in 5% milk–TBS. The blocked membrane was placed in a multiscreen apparatus (BioRad), and approximately 650 μl of hybridoma supernatant was pipetted into individual lanes. Following a 90 minute incubation at room temperature the blot was removed from the apparatus, washed five times for 5 min in TBS-0.5% Tween-20 (TBS-T), and probed with 167 ng/ml of peroxidase-labeled goat antimouse IgG secondary antibody (KPL, Gaithersburg, MD) for 30 min at room temperature. The membrane was washed five times for 5 minutes with TBS-T, and the membrane was developed using the Chemiluminescent Substrate Kit (KPL). The blot was visualized by exposing X-ray film. Positive hybridomas (anti-PSMA reactivity) were identified and selected for further development.

Cloning by Limiting Dilution, Testing Clones, and Purification of Monoclonal Antibody

Primary hybridomas identified by their anti-PSMA reactivity were cloned by limiting dilution. Briefly, the cells were adjusted to a final concentration of 1 cell/ml of RPMI-1640–10% Fetal Clone containing 10⁵ syngeneic thymocytes as a feeder cell population. Two hundred μl of cell suspension was pipetted into wells of

sterile 96-well microtiter plates and cultured for 7–10 days, or until single colonies of cells were visible. Wells containing single colonies were picked, and the clones were expanded in 24-well plates. Supernatants from the clonal cultures were harvested and tested for anti-PSMA reactivity in the Western blot assay described above. Clones producing anti-PSMA monoclonal antibody were expanded, and the cells were used for the generation of high titer ascites fluid. The monoclonal antibody 3F5.4G6, an IgM class anti-PSMA antibody, was purified from ascites fluid using the ImmunoPure IgM Purification Kit (Pierce, Rockford, IL).

Immunoprecipitation of PSMA from LNCaP Tumor Cells Using 3F5.4G6 Monoclonal Antibody

Approximately 10×10^6 LNCaP tumor cells were incubated with 1 ml of NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) for 30 min at 4°C. The lysate was centrifuged at 12,000 rpm for 5 min to remove cellular debris and the resultant supernatant was precleared by incubation with 50 µl of normal mouse serum (Sigma) for 30 min followed by the addition of 60 µl of a 20% suspension of antimouse IgM-coupled agarose beads (Sigma). Following 1-hr incubation at 4°C, the supernatant was centrifuged at 12,000 rpm to remove the beads, the resultant supernatant was used in an immunoprecipitation protocol with 3F5.4G6 monoclonal antibody. Ten µg of purified 3F5.4G6 monoclonal antibody was added to the supernatant and incubated for 1 hr at 4°C. One hundred µl of a 10% suspension of antimouse IgM-coupled agarose beads was added, and the supernatant was incubated for an additional hour at 4°C. The samples were centrifuged at 12,000 rpm, and the agarose beads were washed three times with NP-40 lysis buffer. The agarose beads were resuspended in 30 µl of sample buffer (SDS reducing buffer) and heated for 10 min at 95°C. Following a brief centrifugation at 12,000 rpm, the sample was run on an 8.5% SDS–PAGE gel, and the separated proteins were electroblotted as described above. A Western blot assay as described above was performed on the samples using the PSMA-specific monoclonal antibody 7E11.C5 as the reporting antibody.

In additional controlled studies, the 9H10 monoclonal antibody was employed. This antibody reacts with an unknown protein on the surface of only LN-CaP cells as was initially described, in contrast to 7E11.C5, which reacts to prostate epithelial cells [3].

RESULTS AND DISCUSSION

PSMA is a membrane-bound glycoprotein that shows high tissue specificity for prostatic tissues.

7E11.C5   3F5.4G6

Fig. I.   Western blot assay of LNCaP lysate using 7E11.C5 (lane 1) and 3F5.4G6 (lane 2) monoclonal antibodies and developed with HRP-anti-IgM secondary antibody. It should be noted that 3F5.4G6 recognizes a protein of M_r 120 kDa, which is similar, if not identical, to the protein recognized by 7E11.C5. 3F5.4G6 monoclonal antibody also recognizes a protein of M_r 110 kDa corresponding to the protein PSM'. It should be noted that 7E11.C5 does not recognize PSM' because the epitope of 7E11.C5 monoclonal antibody is N-terminal (amino acid 1'7) and is not found in PSM' because PSM' is a truncated version of PSMA and does not contain the initial 57 amino acids of PSMA. Thus, 3F5.4G6 recognizes the C terminal portion of the protein (amino acid 716–723) and specifically the extracellular domain of PSMA and PSM'.

PSMA was originally defined and its tissue expression characterized based upon the reactivity of the monoclonal antibody 7E11.C5 [3]. These results indicated high specificity for prostatic tissues and an increased expression of the 7E11.C5 antibody in the serum of prostatic cancer patients compared to normal individuals [2,3]. Further studies have confirmed this observation [1,4]. Other studies suggest that PSMA expression in tumors is down-regulated by steroids such as 5α-dihydrotestosterone [9,10]. This behavior is consistent with the elevated expression of PSMA in hormone-refractory tumors. Thus, the results indicate the antigen are almost entirely prostate specific in humans; furthermore, they may be a marker for aggressive clones of prostate cancer cells due to its increased expression in hormone-resistant tumors [1–4]. Figure 1 illustrates on Western blot the reactivity of 7E11.C5 with PSMA in LNCaP cells. It should be noted that monoclonal antibody 9H10 does not recognize a protein (i.e., PSMA) of M_r 110–120 kDa in LNCaP cells, but rather recognizes a protein of M_r 30 kDa of unknown identity.

Using the 7E11.C5 antibody as a probe, Israeli et al. [9] cloned a cDNA from LNCaP cells that encodes a 750-amino acid membrane protein. The deduced amino acid sequence defines a type II transmembrane protein composed of a short cytoplasmic domain, a membrane-spanning domain, and an extracellular domain, a portion of which has high sequence homology to transferrin receptor [9]. Further work has shown that the protein epitope to which the 7E11.C5 antibody binds is composed of the N-terminal amino acids of the protein located in the cytoplasmic domain [5,6]. That is, a "sixmer" composed of the first six amino acids from the N-terminal of PSMA was the

PROSTATE SPECIFIC MEMBRANE ANTIGEN

Fig. 2. Topographical representation of PSMA regions in relation to Hopp and Woods antigen prediction. A recent paper reported a rat brain partial cDNA clone of NAALADase which possesses a high degree of identity to the 3' end of the PSMA cDNA. The putative NAALADase protein is highly homologous to a region of the extracellular domain of PSMA.

smallest peptide element recognized by 7E11.C5. No binding to any other peptide element not containing this terminal sequence was reported [5,6].

A variant of PSMA resulting from alternative splicing has been deduced from reverse transcriptase polymerase chain reaction (RT-PCR) studies and RNase protection assays [9]. This variant, designated PSM', is missing the first 57 amino acids of PSMA and was reported to predominate over PSMA in normal prostatic tissues while the reverse relationship was true in the case of prostatic carcinomas [10]. Thus, the PSM' protein is not recognized by the 7E11.C5 antibody (Fig. 1), and data supporting the physiological expression of PSM' to date rely solely on studies of the nature of mRNA species expressed in prostatic cells and tissues.

We have been interested in expression of PSMA as a possible marker for disease progression, particularly in later stages of the disease. Efforts have so far focused on development of serodiagnostic assays relying on the 7E11.C5 antibody for detection in a quantitative Western blot assay with serum. We now report, herein, the preparation of a monoclonal antibody designated 3F5.4G6 that is specific for an 8-amino acid region located near the C-terminal portion of the molecule (aa 716–723). An analysis of antigenic determinants based upon the Hopp and Woods algorithm demonstrated the antigenic potential of this protein region [11] (Fig. 2). Its presence in

the extracellular domain near the C-terminal of the protein makes this determinant ideal for application to a sandwich-type immunoassay or to detect the presence of PSM' in the serum.

The results presented indicate that the 3F5.4G6 monoclonal antibody reacts specifically with the same protein species as 7E11.C5 in Western blots with LN-CaP cell crude lysates (Fig. 3). Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species, which in both cases is reactive with the reciprocal antibody in Western blot analyses (Fig. 3). Thus, it can be concluded that the 3F5.4G6 antibody recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. The 3F5.4G6 antibody is thus well suited for use in a sandwich immunoassay as either a capture or detection antibody paired with the 7E11.C5 antibody. Current work is focused on this strategy.

The 3F5.4G6 antibody, in addition to its binding to the PSMA protein, cross-reacted with a protein species which correlates in size with the PSM' variant present in Western blots of LNCaP cell lysates (Fig. 3) by virtue of its specificity for a common epitope in the extracellular domain of both protein forms [10]. This, for the first time, provides direct evidence of the expression of PSM' at the protein level and provides a possible means for differential quantitation of each protein form in serum. 3F5.4G6 antibody also recog-

A

1 2 3 4   5 6 7 8

B

1 2

199—   199—

199—

←— PSMA —→

87—   87—

87—   ←— PSMA

48—   48—

48—

7E11.C5   3F5.4G6   3F5.4G6

Fig. 3. A: Demonstration that the 7E11.C5 and 3F5.4G6 monoclonal antibodies recognize identical proteins and that 3F5.4G6 recognizes an additional protein corresponding to PSM'. LNCaP lysate was initially immunoprecipitated with 7E11.C5 monoclonal antibody and the immunoprecipitated material separated on SDS gels and probed in a Western blot assay with either 7E11.C5 (lanes 1–4) or with 3F5.4G6 (lanes 5–8) monoclonal antibodies. Lanes 1, 5, crude LNCaP lysate (0.05 μg protein/well); lanes 2, 6, precleared LNCaP lysate (0.05 μg protein/well); lanes 3, 7, material that immunoprecipitated with 7E11.C5 monoclonal antibody; lanes 4, 8, proteins left in the previously immunoprecipitated LNCaP lysate. It should be noted that 7E11.C5 immunoprecipitated a protein of M, 120 kDa that was recognized not only by 7E11.C5 (lane 3), but also by 3F5.4G6 (lane 7). It should also be noted that present in the supernatant after 7E11.C5 immunoprecipitation was

a protein recognized by 3F5.4G6 (lane 8), but not by 7E11.C5 (lane 4), and that corresponds to PSM'. Thus, 7E11.C5 does not recognize PSM' (i.e., the epitope of 7E11.C5 is amino acids 1–7 of PSMA while the epitope of 3F5.4G6 is amino acid 716–723) and therefore PSM' should remain in the lysate of a 7E11.C5 immunoprecipitated LNCaP lysate and subsequently recognized by 3F5.4G6. B: Demonstration that monoclonal antibody 7E11.C5 and monoclonal antibody 3F5.4G6 recognize identical proteins. PSMA from an LNCaP lysate was immunoprecipitated by monoclonal antibody 3F5.4G6, the proteins in the immunoprecipitate separated on a SDS gel, the proteins transferred to Immobilon P and probed in a Western blot with monoclonal antibody 7E11.C5. Lane 1, LNCaP lysate control; lane 2, 3F5.4G6 immunoprecipitation of 2.5 μg LNCaP lysate and Western blot with 7E11.C5 monoclonal antibody.

nized PSMA in the serum of prostate cancer patients (stage D2; Fig. 4), illustrating its utility in detection of PSMA in the serum of prostate cancer patients.

Studies of the expression of PSMA in the serum of both normal individuals and prostatic cancer patients has provided conflicting results at two laboratories [1–6]. Troyer et al. [5,6] reported evidence suggesting that the binding of 7E11.C5 to Western blots of serum specimens was nonspecific in that the peptide containing the antibody epitope (N1–19) was ineffective in inhibiting antibody binding to a protein species migrating in the same region as PSMA. These studies used differing conditions than was previously reported by this laboratory wherein serum expression was demonstrated [1–4]. The results presented in Figure 5 using the procedures we originally described indicate that 7E11.C5 binding to Western blots of both LNCaP cell lysates and human serum is specific and not due to nonspecific binding of secondary antibody. No labeling of any protein band corresponding to PSMA was detected in the absence of the 7E11.C5 antibody, even after extended exposure of the blot to X-ray film. This evidence strongly supports the presence of PSMA in human serum and is consistent with our earlier results demonstrating elevations in serum PSMA in prostatic cancer patients in clinical progression. In our results, we have also failed to demonstrate PSMA protein in normal human brain tissue, in contrast to a report by Troyer and colleagues, which has

1 2   3 4

███████   ███████ ←— PSMA

7E11.C5   3F5.4G6

Fig. 4. Demonstration by Western blot of PSMA in serum of prostate cancer patients (stage D2) using monoclonal antibody 7E11.C5 (lanes 1, 2) and demonstration of the recognition of PSMA in the same prostate cancer patient by monoclonal antibody 3F5.4G6 (lanes 3, 4). 0.44 μg serum protein was loaded in each well and protein separated on SDS gels as described in Materials and Methods.

the same methodological limitations noted in Figure 5 [5,6]. In addition, studies using the 9H10 monoclonal antibody have failed to show that it interacts with 3F5.4G6 or 7E11.C5 antigen [3].

Moreover, 3F5.4G6 reactivity was not detected in DU-145 or PC3 cell lines—only in LNCaP, as is the case with 7E11.C5 [3]. There is, however, a most recent report demonstrating a clear nucleotide sequence identity for a region of the extra-cellular portion of PSMA (i.e., in the PSM' distal region) which possesses properties of NAALADase membrane hydrolase (figure 2).[12] We are conducting further work to clarify the molecular characteristics of the NAALADase region to 3F5.4G6 and 7E11.C5. Nevertheless, the availability of the 3F5.4G6 antibody for application in a sandwich immunoassay will simplify the detection of serum PSMA and provide a potentially useful clinical tool for monitoring prostatic cancer patients.
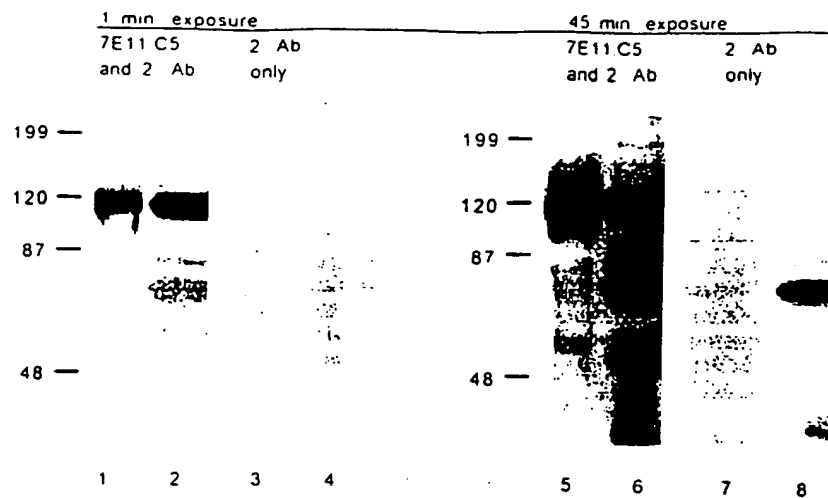
Fig. 5. Demonstration that recognition of a protein in LNCaP lysate (lanes 1, 5) and in the serum of cancer patients (lanes 2, 6) by 7E11.C5 is specific to the primary antibody 7E11.C5 and not due to nonspecific recognition by secondary antibody used to report primary antibody binding to proteins. LNCaP lysate (0.05 µg protein/well; lanes 1,3,5,7) or prostate cancer patient serum (0.44 µg protein/well; lanes 2,4,6,8) was separated on SDS gels as previously described and transferred to Immobilon P paper and processed for Western blot as follows. Lanes 1,2,5,6 were probed with 7E11.C5 monoclonal antibody and then with secondary antibody (i.e., goat antimouse IgG) or with secondary antibody only (lanes 3,4,7,8). The film was exposed for either the routine 1 min (lanes 1–4) or overexposed for a period of 45 min (lanes 5–8). These results demonstrate that the recognition of bands by 7E11.C5 is specific for the primary antibody, and not due to nonspecific binding of secondary or reporting antibody. It should be noted that the same secondary antibody is used with monoclonal 3F5.4G6 as with 7E11.C5, and therefore the 3F5.4G6 monoclonal antibody is specific for PSMA and PSM' as illustrated in Figure 4, 6, 7 and is not due to nonspecific binding of the secondary antibody to proteins in the lysate.

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Antibodies
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6

Serum contains many different types of antibodies that are specific for many different antigens. Even in hyperimmune animals, seldom are more than one-tenth of the circulating antibodies specific for one antigen. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production.

The first isolation of a homogeneous population of antibodies came from studies of B-cell tumors. Clonal populations of these cells can be propagated as tumors in animals or grown in tissue culture. Because all of the antibodies secreted by a B-cell clone are identical, these tumor cells provide a source of homogeneous antibodies. Unfortunately, B-cell tumors secreting antibodies of a predefined specificity cannot be isolated conveniently.

In the animal, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells cannot be grown in tissue culture, they cannot be used as an in vitro source of antibodies. Köhler and Milstein (1975) developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell, isolated from an immunized animal, is fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or *hybridomas* can be maintained in vitro and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as *monoclonal antibodies*.

Monoclonal antibodies are powerful immunochemical tools

The usefulness of monoclonal antibodies stems from three characteristics—their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. The production of monoclonal antibodies allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope. Hybridoma cell lines also provide an unlimited supply of antibodies. Even the most farsighted researchers have found that large supplies of valuable antisera eventually run out. Hybridomas overcome these difficulties. In addition, one unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single-cell cloned prior to use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens.

Hybridomas secreting monoclonal antibodies specific for a wide range of epitopes have been prepared. Any substance that can elicit a humoral response can be used to prepare monoclonal antibodies. Their

specificities range from proteins to carbohydrates to nucleic acids. However, monoclonal antibodies are often more time-consuming and costly to prepare than polyclonal antibodies, and they are not necessarily the best choice for certain immunochemical techniques. In theory, either as single antibody preparations or as pools, monoclonal antibodies can be used for all of the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job. Researchers should be certain that they need these types of reagents before they begin constructing hybridoma cell lines. Table 6.1 summarizes some of the uses of antibodies and some general suggestions for choosing the best reagents.

Hybridomas are immortal somatic cell hybrids that secrete antibodies

In the early 1970s, a number of research groups worked on different methods to extend the life span of antibody-secreting cells in vitro. For murine cells, the practical aspects of this goal were solved by applying techniques used in somatic cell genetics. By fusing two cells, each having properties necessary for a successful hybrid cell line, Köhler and Milstein (1975) showed that antibody-secreting cell lines could be established routinely and maintained in vitro. The two cells that are commonly used as partners in these fusions are antibody-secreting cells isolated from immunized animals and myeloma cells. The myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody-secreting cells provide the functional immunoglobulin genes.

Early work solved the three technical problems for achieving a successful fusion: (1) finding appropriate fusion partners, (2) defining conditions for efficient fusion, and (3) choosing an appropriate system to select for hybrid cells against the background of unfused cells.

TABLE 6.1
Immunochemical Techniques, Polyclonal versus Monoclonal Antibodies

Technique	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
Cell Staining	Usually good	Antibody dependent	Excellent
Immunoprecipitation	Usually good	Antibody dependent	Excellent
Immunoblots	Usually good	Antibody dependent	Excellent
Immunoaffinity Purification	Poor	Antibody dependent	Poor
Immunoassays			
Labeled Antibody	Difficult	Good	Excellent
Labeled Antigen	Usually good	Antibody dependent	Excellent

Myelomas from BALB/c mice are good cells for fusion

Myelomas can be induced in a few strains of mice by injecting mineral oil into the peritoneum. Many of the first examples of these myelomas were isolated from BALB/c mice by Potter (1972), and these cells are referred to by the abbreviation MOPC (for mineral oil plasmacytoma). Derivatives of BALB/c myelomas have become the most commonly used partners for fusions. Table 6.2 lists some of the myeloma cell lines used for hybridoma construction. Myelomas have all the cellular machinery necessary for the secretion of antibodies, and many secrete these proteins. To avoid the production of hybridomas that secrete more than one type of antibody, myelomas that are used for fusions have been selected for the lack of production of functional antibodies. Figure 6.1 shows the derivation of many of the commonly used myeloma cell lines.

The other cell for the fusion is isolated from immunized animals. These cells must carry the rearranged immunoglobulin genes that specify the desired antibody. Because of the difficulties in purifying cells that can serve as appropriate partners, fusions are normally performed with a mixed population of cells isolated from a lymphoid organ of the immunized animal. Although a number of studies have helped to characterize the nature of this B-cell-derived partner, the exact state of differentiation of this cell is still unclear.

Hybridomas can be prepared by fusing myelomas and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore, fusions are normally done with cells from the same species. All commonly used mouse strains can serve as successful fusion partners with BALB/c myelomas; however, immunizations are normally done in BALB/c mice, as this allows the resulting hybridomas to be grown as tumors in this mouse strain.

Polyethylene glycol is the most commonly used agent to fuse mammalian cells

In theory, the fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In practice, hybridoma fusions became routine after the introduction of the use of polyethylene glycol (PEG). The use of PEG as a fusing agent for mammalian cells was first demonstrated by Pontecorvo (1975), and was quickly adopted by somatic cell geneticists. PEG is the method of choice for hybridoma production, allowing the rapid and manageable fusion of mammalian cells.

PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells.

TABLE 6.2
Myeloma Cell Lines Used as Fusion Parents

Cell line	Reference	Derived from	Chains expressed	Secreting	Comments
Mouse Lines					
P3-X63Ag8	Köhler and Milstein (1975)	P3K	$\gamma 1, \kappa$	IgG$_1$	Not recommended
X63Ag8.653	Kearney et al. (1979)	P3-X63Ag8	None	No	Recommended
Sp2/0-Ag14	Köhler and Milstein (1976) Shulman et al. (1978)	P3-X63Ag8 × BALB/c	None	No	Recommended
FO	de St. Groth and Scheidegger (1980)	Sp2/0-Ag14	None	No	Recommended
NSI/1-Ag4-1	Köhler et al. (1976)	P3-X63Ag8	Kappa	No	Recommended
NSO/1	Galfre and Milstein (1981)	NSI/1-Ag4-1	None	No	Recommended
FOX-NY	Taggart and Samloff (1984)	NSI/1-Ag4-1	Kappa (?)	No	
Rat Lines					
Y3-Ag1.2.3	Galfre et al. (1979)	Y3	Kappa	No	Not recommended
YB2/0	Kilmartin et al. (1982)	YB2/3HL	None	No	Recommended
IR983F	Bazin (1982)	LOU/c rats	None	No	Recommended

Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy- or light-chain gene is lost, production of the antibody will stop. In a culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer and will result in unstable lines. If the chromosome that is lost contains a gene used in drug selection (see below), then the growth of the hybridoma will be unstable, and cells will continue to die during selection. In practice, the selection for the stable segregation of the drug selection marker is so strong that within a short time the hybridoma is either lost completely or a variant is isolated that stably retains the selectable marker.
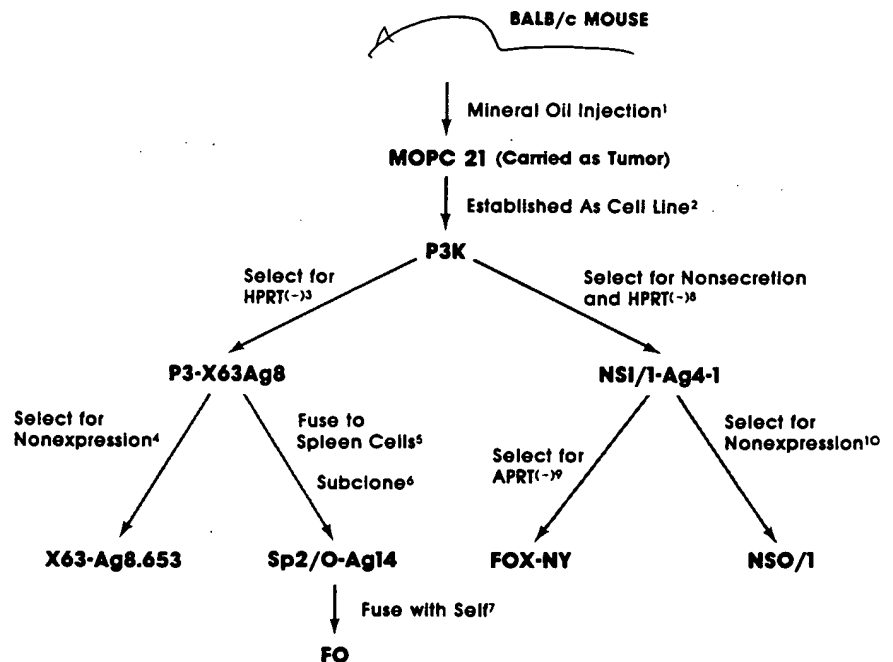

FIGURE 6.1

Myeloma family tree. [1]Potter (1972); [2]Horibata and Harris (1970); [3]Kohler and Milstein (1975); [4]Kearney et al. (1979); [5]Kohler and Milstein (1975); [6]Shulman et al. (1978); [7]de St. Groth and Scheidegger (1980); [8]Kohler et al. (1976); [9]Taggart and Samloff (1982); [10]Galfre and Milstein (1981).

Unfused myeloma cells are eliminated by drug selection

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in the culture. The cells from the immunized animal do not continue to grow in tissue culture, and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed. Most hybridoma constructions achieve this by drug selection. Commonly, the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (first reported by Littlefield 1964). For example, selection with 8-azaguanine often yields a cell line harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compound that blocks the de novo nucleotide synthesis pathway will force cells to use the salvage pathway. Cells containing a nonfunctional HPRT protein will die in these conditions. Hybrids between myelomas with a nonfunctional HPRT and cells with a functional HPRT will be able to grow. Selections are commonly done with aminopterin, methotrexate, or azaserine (Fig. 6.2).
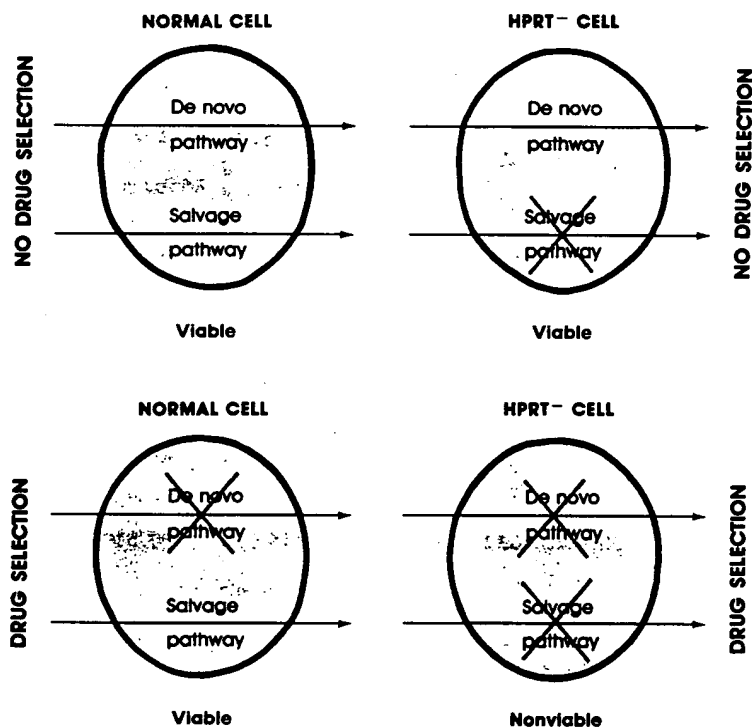
FIGURE 6.2A

Pathways of nucleotide synthesis.

NO DRUG SELECTION

MYELOMA (HPRT⁻)

Viable

CELL FUSION

B CELL

Nonviable (in tissue culture)

NO DRUG SELECTION

DRUG SELECTION

MYELOMA (HPRT⁻)

Nonviable

Viable

B CELL

Nonviable (in tissue culture)

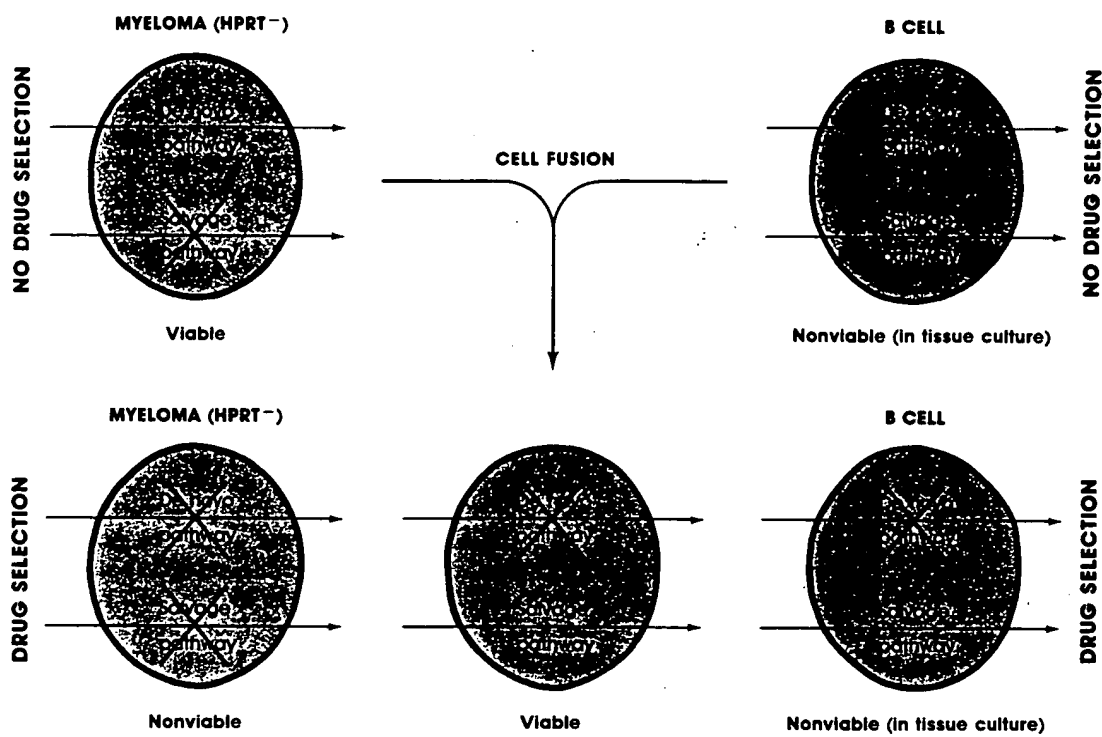**DRUG SELECTION**

FIGURE 6.2B
Drug selection for viable hybridomas.

PRODUCTION OF MONOCLONAL ANTIBODIES

Although hybridomas can be prepared from animals other than mice, all of the techniques below use mice as examples. Similar techniques can be used for fusions of rat myelomas and rat antibody-secreting cells. More specialized fusions using interspecies crosses or human cells are discussed briefly on p. 240 and 241, respectively.

Stages of Hybridoma Production

Figure 6.3 outlines the steps in the production of monoclonal antibodies. Animals are injected with an antigen preparation, and once a good humoral response has appeared in the immunized animal, an appropriate screening procedure is developed. The sera from test bleeds are used to develop and validate the screening procedure. After an appropriate screen has been established, the actual production of the hybridomas can begin. Several days prior to the fusion, animals are boosted with a sample of the antigen. For the fusion, antibody-secreting cells are prepared from the immunized animal, mixed with the myeloma cells, and fused. After the fusion, cells are diluted in selective medium and plated in multiwell tissue culture dishes. Hybridomas are ready to test beginning about 1 week after the fusion. Cells from positive wells are grown and then single-cell cloned. Hybridoma production seldom takes less than 2 months from start to finish, and it can take well over a year. It is convenient to divide the production of monoclonal antibodies into three stages: (1) immunizing mice, (2) developing the screening procedure, and (3) producing hybridomas. Any one of these stages may proceed very quickly, but all have inherent problems that should be considered prior to the start of the project, and these areas are discussed separately below.

IMMUNIZATION

Primary Immunization

2 Weeks

Boost

10 Days

Test Bleed ⊖ ⊕

1 Month-1 Year

SCREEN DEVELOPMENT

Develop and Test Screening Method

2 Weeks Minimum

Final Boost

3 Days

Fuse

~1 Week

Screen ⊕

~1 Month

HYBRIDOMA PRODUCTION

Expand and Freeze

Single Cell Clone

~1 Week

Some ⊕

Screen ⊕

All
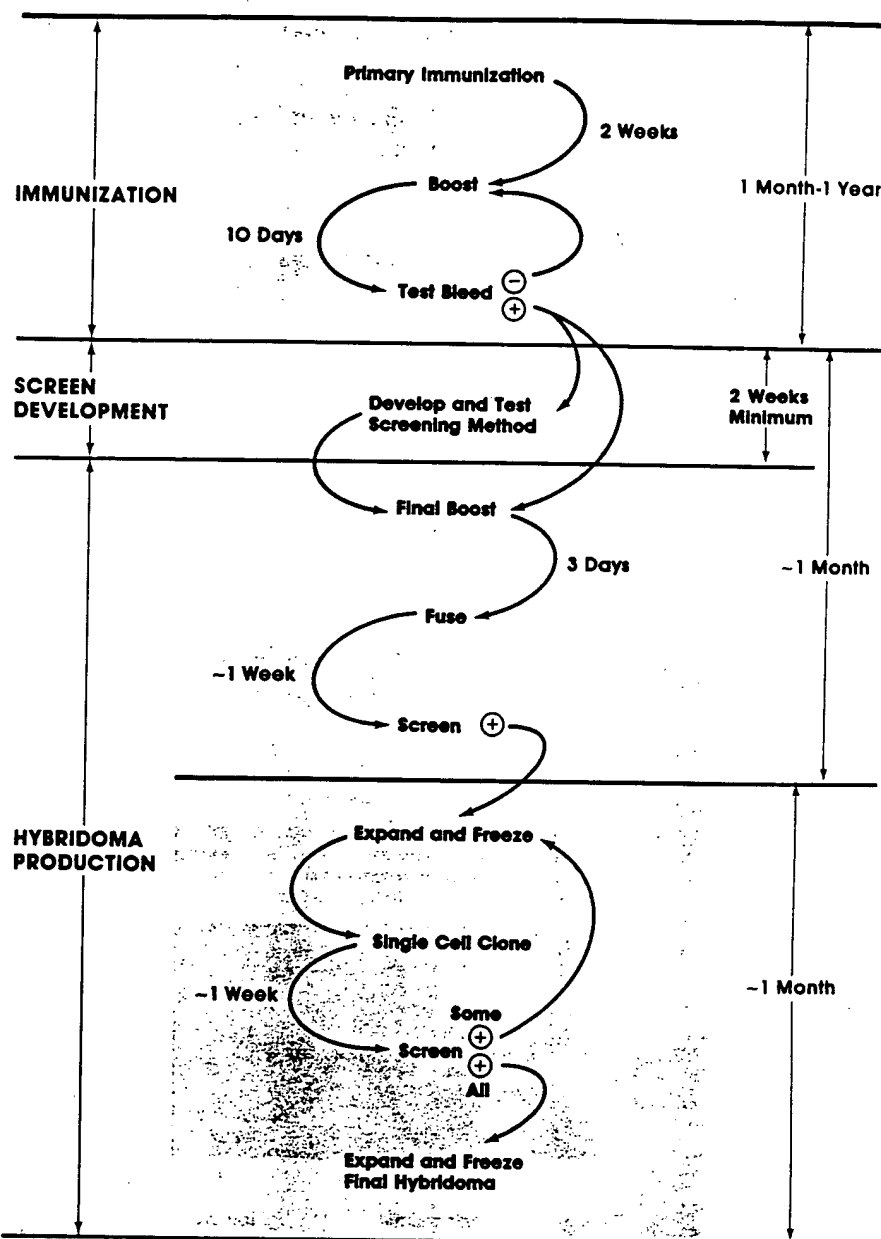
Expand and Freeze Final Hybridoma

~1 Month

FIGURE 6.3
Stages of hybridoma production.

■ IMMUNIZING MICE

Figure 6.4 shows a typical antibody response to multiple injections with a good immunogen. Also included in this figure is a description of the characteristics of a typical monoclonal antibody that might be isolated following one of the immunizations. Although this simplified view can only serve as a rough guide, it does give an indication of the potential time frame for the production of antibodies with particular properties.

Chapters 4 and 5 discuss in detail both the theoretical and practical aspects of immunizing laboratory animals.

IgM	IgG	IgG	IgG
Multivalent, Low affinity, Non-protein A binding	Bivalent, Low to medium affinity, Protein A binding	Bivalent, Medium to high affinity, Protein A binding	Bivalent, High affinity, Protein A binding

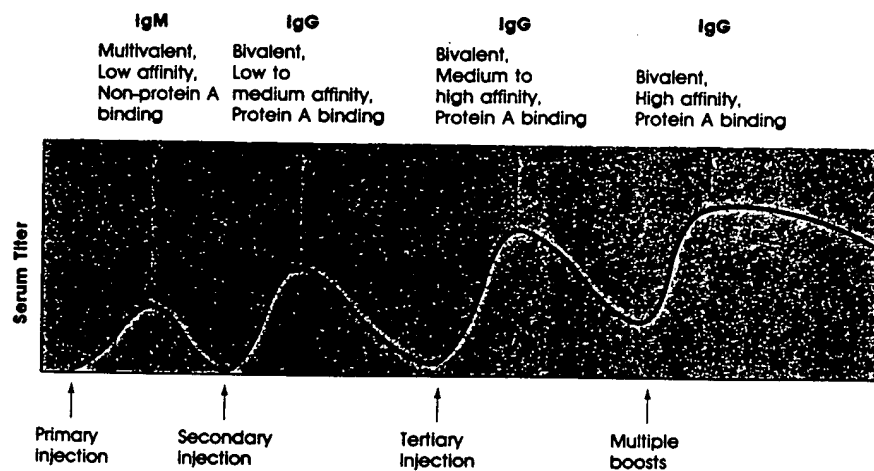Primary Injection   Secondary Injection   Tertiary Injection   Multiple boosts

FIGURE 6.4
Kinetics of a typical immune response.

■ Dose and Form of the Antigen

The amount of antigen necessary to induce a good immune response will depend on the individual antigen and host animal (see Chapter 5). Suggested doses for mice are summarized in Table 6.3.

Soluble Proteins

Soluble protein antigens can yield strong responses and good monoclonal antibodies with doses of as low as 1 μg/injection. More commonly, injections are adjusted to deliver 10–20 μg. If the antigen is available in large quantities, 50 μg should be used. Except for special cases it is seldom worthwhile to use more than 200 μg of a protein antigen per injection. Even if the antigen is not pure, the total dose should not normally exceed 500 μg. When highly conserved proteins are being used to raise antibodies, it is often necessary to modify these antigens prior to injection. This can be done by covalently adding small immunogenic haptens to proteins. Modifying proteins by binding them to large immunogenic proteins such as the hemocyanins has also been shown to be an effective way of breaking T cell tolerance. These methods are discussed in detail in Chapter 5.

TABLE 6.3
Suggested Doses of Immunogens for Mice

Form of antigen	Examples	Primary injections and boosts			Final boosts		
		Possible routes	Dose	Adjuvant	Possible routes	Dose	Adjuvant
Soluble Proteins	Enzymes Carrier proteins conjugated with peptides Immune complexes	ip[a] sc[b]	5–50 µg	+	iv[c]	5–50 µg	–
Particulate Proteins	Viruses (killed) Yeast (killed) Bacteria (killed) Structural proteins	ip sc	5–50 µg	+	iv	5–50 µg	–
Insoluble Proteins	Bacterially produced from inclusion bodies Immunopurified proteins bound to beads	ip sc	5–50 µg	+	ip	5–50 µg	–
Live Cells	Mammalian cells	ip	10^5–10^7 cells	–	iv	10^6 cells	–
Live Tumorigenic Cells	Oncogenic mammalian cells	ip sc	10^4–10^6 cells	–	iv	10^6 cells	–
Carbohydrates	Polysaccharides Glycoproteins	ip sc	10–50 µg	+/–	iv	10–50 µg	–
Nucleic Acids	Carrier proteins conjugated with N.A.	ip	10–50 µg	+	iv	10–50 µg	–

[a] Intraperitoneal.
[b] Subcutaneous.
[c] Intravenous.

Particulate Proteins

In general, particulate antigens make excellent immunogens, because they are readily phagocytosed (see Chapter 4). Soluble proteins may be converted to particulate antigens by self-polymerization or by binding them to solid substrates such as agarose beads (p. 528). Large insoluble antigens should not be injected intravenously (iv) due the possible development of embolisms.

Proteins Produced by Overexpression

Recent advances in recombinant DNA technology have made the production of many protein antigens simple. Overexpression of fusion proteins or full-length polypeptide chains using both prokaryotic and eukaryotic vectors has become routine. These proteins are often excellent antigens and can be produced in large quantities. They normally present few problems for the production of monoclonal antibodies. These proteins can be purified and injected as soluble or insoluble antigens (p. 88).

Synthetic Peptides

A second source of immunogens, based on the availability of a coding sequence, is the in vitro synthesis of peptides. Synthetic peptides, when coupled to carrier proteins such as bovine serum albumin or keyhole limpet hemocyanin, normally elicit a strong humoral response. Constructing these carrier complexes and the production of anti-peptide sera are described in Chapter 5 (p. 73). Using peptide–carrier protein complexes for the production of monoclonal antibodies normally is done only for specific reasons. Because these peptides are relatively short, many of the advantages of monoclonal antibody specificity are lost. Monoclonal antibodies do provide two advantages over polyclonal anti-peptide sera. The first is that the source of the antibodies will be unlimited, and the second is that monoclonal antibodies may be more useful in immunoaffinity purifications. Like all immunizations using peptide antigens, the major difficulty will be in preparing antibodies that will bind to the native protein.

Live Cells

A number of studies have used live cells as immunogens for generating antibodies to surface antigens. Except in unusual circumstances, injections of cells should not include live bacteria or yeast. Although mice are normally capable of killing and clearing bacteria and yeast infections, the possibility of infecting an entire mouse colony is too great to risk these types of injections.

Although large numbers of hybridomas have been prepared to surface antigens of mammalian cells, these antibodies may be of low affinity and care should be taken to ensure that the immune response includes antibodies that will be useful in later studies. When raising

antibodies to live tumorigenic cells, it is easy to pass the cells as tumors and thereby eliminate any activity against tissue culture reagents, including proteins in bovine serum.

Nucleic Acids

Nucleic acids normally are not good antigens, and antibodies to them usually are raised against small haptens bound to carrier proteins. Because nucleic acids are weak antigens, it is particularly important to test sera for antibodies that will work in all assays for which the monoclonal antibodies are being raised.

Carbohydrates

Simple carbohydrates usually are weak immunogens. These compounds should be coupled to carrier proteins. Large complex carbohydrates (> 50,000) will induce a moderate response, but often without a secondary response. High doses readily induce tolerance, so the injected amount should be controlled carefully. These immunogens are best injected as a portion of a larger particle, such as a bacterial cell wall

TABLE 6.4
Routes of Injection

Route	Abbreviation	Uses		Maximum volume
		Primary injections and boosts	Final boost	
Intraperitoneal	ip	Good	Fair	0.5 ml
Subcutaneous	sc	Good	Poor	0.2 ml
Intravenous	iv	Poor	Good	0.2 ml
Intramuscular	im	Not recommended for mice		
Intradermal	id	Not recommended for mice		
Lymph node		Special uses		0.1 ml

or equivalent. Coupling these larger carbohydrates to carrier proteins can be beneficial. For glycoproteins, the polypeptide backbone can function as an effective carrier.

■ Route of Inoculation

Table 6.4 gives a summary of the potential routes of introducing an antigen into mice. Most injections for hybridoma production are done in female mice, because they are somewhat easier to handle than male mice.

> Prior to beginning an immunization, contact your local safety and animal committees for advice on animal care and handling, local regulations, and proper procedures for immunization.

Adjuvant	Immunogen requirement	Comments	Route
+/−	Soluble/or insoluble	If used for final boost, wait 5 days before fusion	Intraperitoneal
+/−	Soluble/or insoluble	Local response, Serum levels slower to increase	Subcutaneous
No Freund's	Soluble, Ionic detergent <0.2% Nonionic detergent <0.5% Salt <0.3 M Urea <1 M	Poor for immunizing	Intravenous
			Intramuscular
			Intradermal
No Freund's	Soluble/or insoluble	Good applications for experienced workers	Lymph node

COMMENTS ■ Immunizations

There are several points that are important in designing an immunization regime that will produce the appropriate monoclonal antibodies.

- Choose the appropriate animal or strain for the desired antibody. Important points to consider are (1) tolerance, (2) amount of antigen available, and (3) specific properties (including ease of purification) of the resultant antibodies. (See Chapters 4 and 5 for details.)

  If no preference in the choice of animal is dictated, then start the immunizations in female BALB/c mice (6 weeks old). In general, mice are cheaper to maintain, are easier to handle, and will respond to lower antigen levels than other laboratory animals. In addition, BALB/c × BALB/c hybridomas can be grown as ascites in BALB/c mice (see p. 274). This can be valuable both in the production of large quantities of monoclonal antibodies and in the eradication of contaminating microorganisms from cultures of hybridoma cells grown in vitro.

- Individual animals, even from the same genetic background, will often respond to identical antigen preparations in completely different ways. Therefore, immunizing more than one animal is a major advantage. In addition, laboratory animals occasionally die, so starting immunizations with several animals may save valuable time.

- Hyperimmunization (multiple immunizations with the same antigen) will yield antibodies with higher affinity for the antigen, especially when the immunizations are widely spaced over a period of weeks to months (see Chapter 5). However, multiple immunizations will not continue to increase the number of epitopes that are recognized.

- Except in unusual circumstances, **do not** start the fusion until the serum from the test bleed contains antibodies with the desired specificity. This may mean extensive testing of the serum in a number of assays, but do not expect to recover antibody activities from the fusion that are not found in the test serum.

- If the animal responds weakly or not at all, consult Chapter 5 for suggestions.

INTRAPERITONEAL INJECTIONS—WITH ADJUVANT

Intraperitoneal injections (ip) are the most commonly used method for introducing antigens into mice. Because of the large volume of the peritoneal cavity, the volume of the immunogen can be larger for ip injections than for other sites. Also because the injections do not deliver the antigen directly into the blood system, particulate antigens can be used.

> **Caution** Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. The antigen should be dispersed in approximately 250 μl of buffer. Draw the solution into a 1.0-ml syringe. Draw an equal volume of Freund's adjuvant into a second syringe. For a primary immunization the adjuvant should be complete Freund's (see p. 98). Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria. All other injections should be in incomplete Freund's.

2. Aqueous antigen solutions and oil-based adjuvants are immiscible, but when mixed will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both

FIGURE 6.5
Preparing Freund's adjuvant for injection.

syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the aqueous antigen solution first. Push the mixture between the two syringes until it becomes difficult to continue (approximately 10–20 times). Then push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- or 25- gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

3. Hold the mouse as shown in Figure 6.6. Inject the antigen–adjuvant emulsion into the peritoneal cavity.

NOTES

i. If the volume of the antigen solution is small (100 μl or less), the emulsion between the Freund's and aqueous solutions may be prepared by vortexing or sonicating in a 1.5-ml conical tube.

ii. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes with luer locks are best).

iii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

FIGURE 6.6
Intraperitoneal injection of a mouse.

INTRAPERITONEAL INJECTIONS—WITHOUT ADJUVANTS

Intraperitoneal injections can also be used to deliver live cells into the peritoneal cavity. These immunizations normally are used to introduce live mammalian cells into mice and prepare anti–cell-surface antibodies. In general, adjuvants should not be used.

1. Cells should be washed extensively in PBS or other isotonic solutions prior to injection to remove as many extraneous proteins as possible. For example, many of the components of bovine serum are highly immunogenic, and if they are injected with the cells, can be antigenically dominant. Even washed cells will have a large number of extraneous proteins bound to the plasma membrane. If this remains a problem, it may be necessary to transfer the cells to low serum, serum-free medium, or mouse serum prior to the injection.

2. The cells should be taken up in 500 μl of PBS and injected using a 25-gauge needle. Hold the mouse as shown in Figure 6.6.

3. Inject the cells into the peritoneum.

NOTES

i. Normal doses of mammalian cells will be between 10^5 and 10^7 cells/injection.

ii. Because of potential infections of the mouse colony, injections of live viruses, bacteria, or fungi normally are not recommended. These antigens are commonly killed or inactivated prior to injection.

INTRAPERITONEAL INJECTION—ANTIGEN BOUND TO BEADS

One excellent method to increase the chances of an antigen being phagocytosed is to bind it to beads. Protein antigens can be bound by free amino groups to any of a number of agarose or polyacrylamide beads. The methods for coupling are discussed on p. 528. After coupling the beads are injected ip with adjuvant as described on p. 158. These preparations should never be injected iv because the chance of forming embolisms is too great.

INTRAPERITONEAL INJECTION — NITROCELLULOSE

Protein antigens can be bound to nitrocellulose and injected or implanted into the peritoneum. Two approaches can be used. If the entire piece of nitrocellulose will be implanted, the subcutaneous route is suggested (see p. 166). For intrapertioneal injections, the nitrocellulose should either be fragmented or dissolved.

> **Caution** Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. Incubate a solution of the antigen (no more than 1.0 mg/ml in PBS) with a sheet of nitrocellulose (0.1 ml/cm^2) at room temperature for 1 hr in a humid atmosphere. For some more abundant antigens or partially purified antigens, the proteins can be transferred directly from an SDS-polyacrylamide gel using standard blotting techniques (see p. 479).

2. Wash the sheet three times with PBS.

3. **Either**: Drain the paper well and freeze at −70°C for 10 min (liquid nitrogen also works well). Transfer to a clean, cold mortar and pestle and quickly grind the paper into small pieces. Remove the plunger from a 1.0-ml syringe, and transfer the pieces into the barrel. Use 250 μl of PBS to help in the transfer.

   Or: Allow the nitrocellulose to dry completely. Remove the plunger from a 1.0-ml syringe. Push the nitrocellulose (use pure nitrocellulose without acetate) to the bottom of barrel. Reinsert the plunger and depress completely. Draw up 125 μl of dimethylsulfoxide into the syringe. Alllow to sit at room temperature for 30 min. Draw up 125 μl of PBS.

   Or: Allow the nitrocellulose to dry completely. Remove the plunger from a 1.0-ml syringe. Push the nitrocellulose (use pure nitrocellulose without acetate) to the bottom of the barrel. Push the luer lock of the syringe into a three-way valve with the valve opening to the syringe closed. Add 250 μl of acetone to the syringe. Tap the syringe several times to mix the acetone with the nitrocellulose. Leave the syringe open and allow to dry (several hours). Remove the three-way valve and replace the plunger. Push the plunger to the end and draw 250 μl of PBS into the syringe.

4. Draw 250 μl of Freund's adjuvant into a second syringe. For a primary immunization the adjuvant should be complete Freund's. (Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria.) All other injections should be in incomplete Freund's.

5. The nitrocellulose mixture and the oil-based adjuvant are immiscible, but when mixed will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the PBS solution first. Continue to push the mixture between the two syringes until it becomes difficult (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- to 25-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

6. Hold the mouse as shown in Figure 6.6. Inject the antigen–adjuvant emulsion into the peritoneal cavity.

NOTES

i. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes with luer locks are best).

ii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

SUBCUTANEOUS INJECTIONS—WITH ADJUVANTS

Subcutaneous injections (sc) are used to deliver soluble or insoluble antigens into a local environment that is a good site of lymphoid draining. Maximum volumes for sc injections are about one-fifth the maximum used for ip injections (100 μl compared to 500 μl). Subcutaneous injections normally are done at more than one site to help ensure that the antigen is detected.

> **Caution** Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. The antigen should be dispersed in approximately 50–100 μl per site of injection. Take up into a 1.0-ml syringe. Take up an equal volume of Freund's adjuvant into a second syringe barrel. For a primary immunization the adjuvant should be complete Freund's. (Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria.) All other injections should be in incomplete Freund's.

2. The adjuvant, which is oil based, and the aqueous antigen solutions are immiscible, but with mixing will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both syringes before connecting to the valve. Make sure the connections are securely tightened. Depress the plunger on the syringe containing the aqueous antigen solution first. Continue to push the mixture between the two syringes until it becomes difficult to push (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- or 25-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

3. Hold the mouse as shown in Figure 6.7. Inject approximately 200 μl total under the skin.

NOTES

i. If the volume of the antigen solution is small (100 μl or less), the emulsion between the Freund's and aqueous solutions may be prepared by vortexing or sonicating in a 1.5-ml conical tube.

ii. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes are best).

iii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

SUBCUTANEOUS INJECTIONS—WITHOUT ADJUVANTS

Like the peritoneal injections, subcutaneous (sc) injections that do not use adjuvants normally are used for delivering live cells to the mouse. This route is often used for tumorigenic cells.

1. Cells should be washed carefully prior to injection to remove proteins from the growth medium. For example, many of the components of bovine serum are highly immunogenic, and, if they are injected with the cells, they can be antigenically dominant. Even washed cells will have a large number of extraneous proteins bound to the plasma membrane. If this remains a problem, it may be necessary to transfer the cells to low serum, serum-free medium, or mouse serum prior to the injection.

2. The cells should be resuspended in approximately 100 μl of PBS per site of injection. Use a 25-gauge needle.

3. Hold the mouse as shown in Figure 6.7. Inject under the skin.

NOTES

i. Normal doses of mammalian cells will be between 10^5 and 10^7 cells/injection.

ii. Because of potential infections of the mouse colony, injections of live viruses, bacteria, or fungi normally are not recommended. These antigens are commonly killed or inactivated prior to injection.

FIGURE 6.7
Subcutaneous injection of a mouse.

SUBCUTANEOUS IMPLANTS—NITROCELLULOSE

Protein antigens immobilized on nitrocellulose often make exceptionally good immunogens. This is probably due to their slow release from the paper, thus behaving somewhat like an adjuvant (p. 96). Not all antigens show increased immunogenicity using this methodology, but some do. The antigen is bound to paper and is implanted on the back of the mouse's neck, a location that makes it difficult for the mouse to disturb the surgical clip.

1. Incubate a solution of the antigen (no more than 1.0 mg/ml in PBS) with a sheet of nitrocellulose (0.1 ml/cm^2) at room temperature for 1 hr in a humid atmosphere.

2. Wash the sheet three times with PBS.

3. Anesthetize the mouse by injecting a suitable drug (see Chapter 5, p. 95). For mice, 0.05 ml of Nembutal ip (sodium pentobarbitone, 40–85 mg/kg) is appropriate. The mouse will be ready for the operation in about 1–2 min.

4. Swab the skin on the mouse's back, just below the base of the neck, with alcohol. Raise the skin with a forceps and make a 1.5-cm incision with sterile scissors. Pull the skin from the inner body wall and insert a 0.5-cm^2 piece of the nitrocellulose. Close the incision with a skin clamp and return the mouse to its cage.

5. Take weekly serum samples beginning about 14 days after the implant. About 10 days after the serum titer drops, the final boost can be given. The final boost will still be an iv injection.

INTRAVENOUS INJECTIONS

Intravenous injections (iv) can be used for two purposes. When immunizing mice, their main use will be to deliver the final boost just before a hybridoma fusion (p. 210). However, iv injections also are useful to ensure that the antigen is seen by the immune system. A rapid and strong response can be expected, as the antigen will be collected quickly in the spleen, liver, and lungs. The antigen will be processed quickly and no continued release of the antigen into the immune system can be expected. Consequently, these types of injections produce a short-lived response.

Intravenous injections should never be used as primary injections, and they must not contain large particulate matter. Because the injection will introduce the antigen directly into vital organs, harsh chemicals must be avoided. Similarly, adjuvants such as Freund's should not be used (p. 96).

1. Isolate the mouse in a small cage or container. Heat the mouse with an infrared lamp. This will increase the blood supply to the tail, making the veins easier to inject. Be careful of the length of time the mouse is left under the lamp. If it's too hot for your hand, it's too hot for the mouse.

2. Move the mouse to a restraining device as shown in Figure 6.8.

3. Swab a portion of the tail with alcohol about 1.5 cm below the base. The veins on the tail should be easily visible.

4. Use a 1.0-ml syringe fitted with a 26- or 27-gauge needle. Hold the tail firmly with one hand and guide the needle into one of the veins. Gently draw back on the plunger. If the needle is in the vein, there will be very little resistance and blood will appear in the barrel. If there is strong resistance and no blood appears, the needle is not in the vein. Withdraw the needle and move to a second site or to another vein. This technique may require a little practice before you can hit the vein readily. Practice with injections of PBS.

5. After you are sure the needle is in the vein, slowly deliver the injection. Pause a few seconds, remove the needle, and return the mouse to its cage. The antigen should be in solution and no adjuvant should be included. Except for specialized injections, the maximum amount to give a mouse by this method is 0.2 ml.

NOTES

i. Injection by this route into immunized animals may cause an anaphylactic reaction. This can be prevented by a prior injection of an antihistamine. Contact your local animal committee for guidance.

ii. Solutions for iv injections should not contain high concentrations of denaturing agents and should be free of toxic chemicals such as sodium azide.

FIGURE 6.8
Intravenous injection of a mouse.

INJECTIONS DIRECTLY INTO LYMPHOID ORGANS

These are injections for more specialized delivery of antigens. They are often appropriate for small amounts of antigen and particularly for secondary or later boosts. In theory, these types of injections may be the best methods for giving a final boost, but because they demand more skill, they are not in common use. The two most frequently used sites of injection are the footpad and the spleen.

In general, footpad and spleen injections should be done only for highly specialized antigens and then only after consulting local authorities for the proper protocols.

■ Identifying Individual Mice

Beginning with the first test bleeds, it is essential to mark the mice so that the immune response can be monitored in individuals. There are a number of methods that are currently used to identify mice. These include ear punches, toe clips, and tail markings. If your animal facility has a standard method, consult them for the proper codes. If not, an acceptable method that is not harmful to the mice is to color the toes of their hindlegs with an indelible marker. This procedure is relatively easy, and, because the marks are on the back legs, the mice don't seem to work as hard to remove the markings as on other sites. Even so, the marks need to be reapplied twice a week.

If there is a large difference between the responses in individual mice, it may be worthwhile to isolate individual mice in separate cages to ensure that the proper mouse is given the final boost.

■ Test Bleeds

Except in unusual circumstances, it is seldom worthwhile to fuse antibody-secreting cells from animals that do not have a usable titer of antibodies in their serum. Periodic test bleeds collected from immunized animals should be checked for the desired antibodies. Tests are run conveniently on small batches of serum prepared from tail bleeds of immunized mice.

The test bleed will yield small samples of polyclonal sera. These sera should be tested in assays that will detect the presence of antibodies specific for the antigen. These tests are discussed in detail in Chapters 10–14.

To make appropriate comparisons in these tests, two practical matters need to be considered. First, the test bleeds should always be titered to monitor the development of the response. The appropriate dilutions will depend on the strength of the response and on the type of assay, but in general 1 in 5 or 1 in 10 dilutions will be satisfactory. Second, the proper negative control should be another polyclonal serum and not an unrelated monoclonal antibody. Most often, this negative control will be serum collected either from another uninjected animal or from an animal that has been boosted with an unrelated antigen. Although it is not always necessary, using serum from a test bleed collected before immunization of the animal is the best negative control. These bleeds are known as preimmune sera.

COLLECTING SERA FROM A MOUSE BY TAIL BLEED

1. Isolate the mouse in a small cage or container. Heat the mouse under an infrared lamp for a few minutes. This treatment will increase the blood flow to the tail, but be careful not to hurt the mouse. If it's too hot for your hand, then it's too hot for the mouse. Place the mouse in a restricted space as shown in Figure 6.8.

2. Swab a portion of the tail about 1.5–2 inches from the body with alcohol. Using a sterile scalpel, nick the underside of the tail across one of the lower veins that should be visible. Collect several drops of blood in a tube and return the mouse to its cage.

3. Incubate the blood at 37°C for 1 hr. Flick the tube several times to dislodge the blood clot.

4. Transfer to 4°C for 2 hr or overnight.

5. Spin at 10,000g for 10 min at 4°C.

6. Remove the serum from the cell pellet. Discard the cell pellet and spin the supernatant a second time for 10 min. Remove the serum, being careful to avoid the packed cell pellet.

7. Add sodium azide to 0.02% and test. Any remaining serum may be frozen at −20°C. The yield is approximately 100–200 μl.

⬛ Deciding to Boost Again or to Fuse

Three factors will influence the decision to proceed with the production of monoclonal antibodies. They all are related to the quality and strength of the immune response. First is whether the antibodies recognize the antigen of interest. This is the most straightforward of the factors and the simplest to determine. The second is a complicated set of properties of the antibodies themselves and the strength of the immune response. These properties are manifested as different titers of antibodies and different affinities of the antibody for the antigen. The third factor is the appearance of spurious antibody activities against unrelated antigens.

In many cases the tests will be relatively easy, and the interpretation apparent. First, the sera should be checked for antibodies that bind to the immunogen itself. For example, if a purified antigen is used, sera could easily be tested for activity in a simple antibody capture assay (p. 175), or if whole cells are used then testing for binding to the cell surface should be done first (p. 184). However, if the monoclonal antibodies will be used for tests other than these simple assays, test bleeds should be checked in assays that resemble, as closely as possible, the tests for which the antibodies are being prepared. For many antibodies, the most useful test will be the immunoprecipitation of the antigen (p. 429). This assay is easy when only testing a few samples, and it will identify antibodies that will be useful in a large number of tests that depend on binding to the native antigen. If, however, the antibodies will be used extensively in immunoblot analyses, in immunohistochemical staining, or in other tests in which many antibodies may fail to work, these tests should be run as well.

Second, sera should also be monitored for the concentration of specific antibodies by titering the test bleeds in the appropriate assays. As the immune response matures, higher levels of specific antibodies will be found. However, higher levels of antibodies do not necessarily mean higher affinities. If high affinity is crucial to the intended use of the antibody, the sera should be titered and compared in assays that are sensitive to antibody affinity, such as immunoprecipitation.

The third factor to consider is the appearance of antibody activities against extraneous antigens. This response may be directed against other antigens in your preparations or may be a response to other antigens in the mouse's environment, including invasion by a pathogenic organism. If the mouse is ill, do not proceed with hybridoma construction. Isolate the mouse in a separate cage and allow it to recover before continuing. If a particularly valuable antigen is being used, more care and veterinary help may be needed. If the antibody activities are to contaminating antigens in the immunogen, a decision must be made whether to proceed. In general, making monoclonal antibodies against complex and multicomponent antigens is a very useful way of isolating specific immunochemical probes, particularly when the antigen is difficult to purify further. However, if the response against the other antigens continues to increase without a concomitant strengthening of the response to the desired antigen,

other approaches may need to be taken. Either other mice should be tested (other individuals or other strains) or the antigen may need to be purified further before proceeding.

■ DEVELOPING THE SCREENING METHOD

Because most hybridoma cells grow at approximately the same rate, the tissue culture supernatants from all the fusion wells usually are ready to screen within a few days of one another. This means that screening is normally the most labor-intensive segment of hybridoma production. Care in developing the proper screen will help to keep the amount of work needed to identify positive wells to a minimum.

Approximately 1 week after the fusion, colonies of hybrid cells will be ready to screen. During the screening, samples of tissue culture media are removed from wells that have growing hybridomas and are tested for the presence of the desired antibodies. Successful fusions will produce between 200 and 20,000 hybridoma colonies, with 500–1000 colonies being the norm. Depending on the fusion, individual wells will become ready to screen over a 2- to 6-day period. Typically, the first wells would be ready to screen on day 7 or 8, and most of the wells will need to be screened within the next 4 or 5 days.

A good screening procedure must: (1) reduce the number of cultures that must be maintained to a reasonable level (seldom more than 50 cultures at one time), (2) identify potential positives in 48 hr or less (24 hr or less is ideal), and (3) be easy enough to perform for all the needed wells. Positive wells may be as rare as 1 in 500 or as common as 9 out of 10. Several screening steps can be combined to identify the desired clones, as long as the first screen reduces the tissue culture work to a manageable level. After the first round of screens, handling the tissue culture necessary for 100 wells is difficult for one person, 50 wells is reasonable, and less than 20 is relatively simple.

All screening procedures must be tested and validated before the fusion has begun. After the fusion, there is seldom enough time to try out new ideas or to refine methods. The test bleeds should be used to set up and test the screening assay.

■ Screening Strategies

There are three classes of screening methods, antibody capture assays, antigen capture assays, and functional screens. Currently, most screens are done by either antigen or antibody capture, but as functional assays become easier to use, more fusions will be screened by these methods. Table 6.5 depicts several of the more common screening techniques.

In general, the more antigens in the immunizing injections, the more difficult the screen. Researchers with pure or partially pure antigens should use methods for antibody capture. If the subcellular location of an antigen is known, positive tissue culture supernatants can be identified by cell staining. If the immunizations used complex antigen solutions, procedures such as immunoprecipitation or other antigen capture assays may be the only alternatives.

In addition to the tests described below, any of the assays used for analyzing antigens can be adapted for use as a screen (see Chapters 10–14).
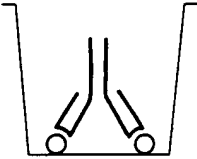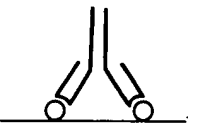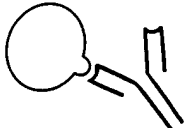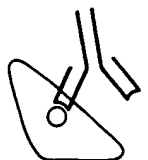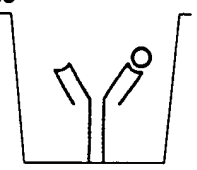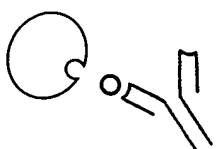
■ Antibody Capture Assays

Antibody capture assays are often the easiest and most convenient of the screening methods. In an antibody capture assay the following sequence takes place: the antigen is bound to a solid substrate, the antibodies in the hybridoma tissue culture supernatant are allowed to bind to the antigen, the unbound antibodies are removed by washing, and then the bound antibodies are detected by a secondary reagent that specifically recognizes the antibody. In this assay the detection method identifies the presence of the antibody, thus determining a positive reaction.

Most antibody capture assays rely on an indirect method of detecting the antibody. This is commonly done with a secondary reagent such as rabbit anti-mouse immunoglobulin antibodies. These antibodies can be purchased from commercial suppliers or can be prepared by injecting purified mouse immunogloblins into rabbits (see p. 622). The rabbit antibodies can be purified, labeled with an easily detectable tag (pp. 288 and 319), and used to determine the presence of mouse monoclonal antibodies.

Alternatively, positives can be located by other reagents that will bind specifically to antibodies. Two proteins that may be used for these reactions are protein A and protein G (see p. 615). Both of these polypeptides are bacterial cell wall proteins that have high affinities for a site in the Fc portion of some antibodies. Protein A and protein G can be purified and labeled with an appropriate tag.

TABLE 6.5
Methods for Screening Hybridoma Fusions

Method	Examples
	PVC wells
Antibody Capture	Nitrocellulose
	Whole cells
	Permeabilized cells
Antigen Capture	Ab/Ag in solution
	Ab/Ag on solid phase
Functional	Blocking
	Depletion

Advantages	Disadvantages

Easy
Rapid
	Need pure or partially
	   pure antigen
	Doesn't discriminate
	   between high- and low-affinity Ab

Relatively easy Doesn't need pure Ag Learn Ag locale	Ag prep can be tedious Doesn't discriminate between high- and low-affinity Ab

Only detect
   high-affinity Ab
	Unless you have
	   pure labeled Ag,
	Assay is tedious
	   and slow

Rapid	Need pure labeled Ag Setting up solid phase is tricky
Ab immediately useful	False positives Potentially tedious

Ab immediately
   useful
Only detect high
   affinity Ab
	Tedious
	Ag must be
	   limiting

ANTIBODY CAPTURE ON NITROCELLULOSE—DOT BLOTS*

If the antigen is a protein that is available in large amounts, dot blots are one of the assays of choice. The antigen is bound directly to the nitrocellulose sheet. Many assays are performed on a single sheet; therefore, the manipulations are simple.

Assays using polyvinylchloride multiwell plates in place of nitrocellulose sheets are good alternatives to dot blots. They are discussed on pp. 180 or 182.

1. A protein solution of at least 1 μg/ml is added to a nitrocellulose sheet at 0.1 ml/cm². Allow the protein to bind to the paper for 1 hr. Higher concentrations of proteins will increase the signal and make screening faster and easier. If the amount of protein is not limiting, concentrations of 10–50 μg/ml should be used. Nitrocellulose can bind approximately 100 μg of protein per cm².

2. Wash the nitrocellulose sheet three times in PBS.

3. Place the sheet in a solution of 3% BSA in PBS with 0.02% sodium azide for 2 hr to overnight. To store the sheet, wash twice in PBS and place at 4°C with 0.02% sodium azide. For long-term storage, shake off excessive moisture from the sheet, cover in plastic wrap, and store at −70°C.

4. Place the wet sheet on a piece of parafilm, and rule with a soft lead pencil in 3-mm squares. Cut off enough paper for the number of assays.

5. Apply 1 μl of the hybridoma tissue culture supernatant to each square. Incubate the nitrocellulose sheet on the parafilm at room temperature in a humid atmosphere for 30 min.

   Along with dilutions of normal mouse serum, include dilutions of the mouse serum from the last test bleed as controls. Dilutions of the test sera are essential to control correctly for the strength of the positive signals. Mouse sera will often contain numerous antibodies to different regions of the antigen and therefore will give a stronger signal than a monoclonal antibody. Therefore, dilutions need to be used to lower the signal. Good monoclonal antibodies will appear 10-fold less potent than good polyclonal sera.

6. Quickly wash the sheet three times with PBS, then wash two times for 5 min each with PBS.

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*Adapted from Sharon et al. (1979); Glenney et al. (1982); Hawkes et al. (1982); Herbrink et al. (1982); Huet et al. (1982); Yen and Webster (1982); and reviewed in Hawkes (1986).

7. Add 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin per 3-mm square in 3% BSA/PBS with 0.02% sodium azide (about 2.0 ml/cm^2).

8. After 30–60 min of incubation with shaking at room temperature, wash extensively with PBS until counts in the wash buffer approach background levels.

9. Cover in plastic wrap and expose to X-ray film with a screen at −70°C.

NOTES

i. The types of bonds that hold protein to nitrocellulose are not known. However, the binding is blocked by oils or other proteins. Wear gloves and use virgin nitrocellulose sheets. For short- or long-term storage, do not leave the paper in buffers containing other proteins. Slow exchange occurs and this will lower the strength of the signal. For long-term storage, freeze the paper in plastic wrap at −70°C.

ii. In all of the antibody capture assays, the method for detecting the antibody can be substituted with other techniques. Most techniques employ either ^{125}I-labeled rabbit anti-mouse immunoglobulins or horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins. For nitrocellulose tests using enzyme-linked reagents, only substrates that yield insoluble products should be used (p. 681). Both enzyme- and ^{125}I-labeled reagents can be purchased from commercial suppliers or prepared in the lab (p. 319). If the detection method uses horseradish peroxidase rather than ^{125}I-labeled rabbit anti-mouse immunoglobulin, sodium azide will block the development of the color.

iii. In all the assays in which proteins are bound to nitrocellulose or polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ANTIBODY CAPTURE IN POLYVINYLCHLORIDE WELLS—
^{125}I DETECTION*

Antibody capture assays in polyvinylchloride (PVC) plates are one of the most commonly used assays. Each well serves as a separate assay chamber, but because they are molded together the manipulations are simple. Two variations of these techniques are listed here, one for ^{125}I detection and one for enzyme-linked assays (p. 182). Both are easy and accurate. The radioimmune assay is easier to quantitate, but the enzyme assay is adequate for most purposes and avoids the problems of radioactive handling.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

5. Fill the wells with 3% BSA/PBS with 0.02% sodium azide. Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of the tissue culture supernatant. Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS containing 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin to each well. Incubate 1 hr at room temperature. (^{125}I-Labeled reagents can be purchased or prepared as described on p. 324.)

*Adapted from Catt and Tregear (1967); Salmon et al. (1969).

10. Wash the plate with PBS until there are no more counts in the wash buffer.

11. Either cut the wells apart and count in a gamma counter or expose the entire plate to film.

NOTES

i. In all the assays in which proteins are bound to nitrocellulose or polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use those that work best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ii. Antigen-coated plates can be prepared in advance and stored. After the blocking solution has been removed, store at $-70°C$.

ANTIBODY CAPTURE IN POLYVINYLCHLORIDE WELLS— ENZYME-LINKED DETECTION*

As an alternative to using ^{125}I-labeled reagents for antibody capture assays in polyvinylchloride (PVC) plates (p. 180), enzyme-linked assays can be employed. The assay is preformed identically to the ^{125}I-labeled assay except the detection methods are changed.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

5. Fill the wells with 3% BSA/PBS (no sodium azide). Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of the tissue culture supernatant. Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS (without sodium azide) containing a dilution of horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin antibody to each well. Incubate 1 hr at room temperature. (Horseradish peroxidase-labeled reagents can be purchased or prepared as described on p. 344. Most commercial reagents should be diluted 1 in 1000 to 1 in 5000. Try several dilutions in preliminary tests and choose the best.)

10. Wash the plate with PBS three times.

*Adapted from Catt and Tregear (1967); Salmon et al. (1969); Envgrall and Perlmann (1972).

11. During the final washes prepare the TMB substrate solution. Dissolve 0.1 mg of 3',3',5',5'-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is sufficient for two 96-well plates. (Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.)

12. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

13. Incubate for 10–30 min at room temperature. Positives appear pale blue.

14. Add 50 μl of stop solution, 1 M H_2SO_4, to every well. Positives now appear bright yellow. To quantitate the binding, read the results at 450 nm.

NOTES

i. In all the assays in which proteins are bound to polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you.

ii. Antigen-coated plates can be prepared in advance and stored. After the blocking solution has been removed, store at -70°C.

iii. Do not include sodium azide in solutions when horseradish peroxidase is used for detection.

ANTIBODY CAPTURE ON WHOLE CELLS—
CELL-SURFACE BINDING*

If the subcellular location of an antigen is known, cell staining assays can be used to screen hybridoma tissue culture supernatants. Two assays are given here, one for cell-surface screening using detection with ^{125}I-labeled reagents and a second for internal localization using enzyme-labeled reagents. Also, any of the techniques for cell staining in Chapter 10 can be adapted for screening.

1. Prepare a suspension of target cells at $1-2 \times 10^6$ cells/ml in 1% BSA/PBS with 0.1% sodium azide. If the cell pellets are particularly difficult to see, add a drop of a suspension of red blood cells or other colored particle that will not interfere with the assay.

2. Add 100 μl of the cell suspension to the wells of a V-bottomed polyvinylchloride (PVC) plate.

3. Centrifuge the PVC plate for 5 min at 400g. Many centrifuge manufacturers supply suitable plate carriers for these types of assays.

4. Carefully remove the supernatants by aspiration.

5. Resuspend the cell pellet without adding any buffer by tapping the plate or by using a microshaker. Dispersing the cell pellet is important for the rapid binding of antibody to the surface antigens.

6. Add 50 μl of tissue culture supernatant. Incubate at 4°C for 1 hr with periodic shaking.

7. Centrifuge the PVC plate for 5 min at 400g. Remove the tissue culture supernatant by aspiration.

8. Wash the cells twice by resuspending in 200 μl of ice cold 1% BSA/PBS with 0.1% sodium azide and centrifuging the plate.

9. Add 50 μl of ice-cold 1% BSA/PBS with 0.1% sodium azide containing 50,000 cpm of ^{125}I-labeled Fab fragment of rabbit anti-mouse immunoglobulin antibodies. Incubate at 4°C for 90 min with periodic shaking. If the target cells do not have a receptor for the Fc portion of immunoglobulins, then ^{125}I-labeled rabbit anti-mouse immunoglobulin antibody may be used in place of the Fab fragment.

*Goldstein et al. (1973); Jensenius and Williams (1974); Morris and Williams (1975); Welsh et al. (1975); Galfre et al. (1977); Williams (1977).

10. Centrifuge the PVC plate as before. Wash the cells three times with ice-cold 1% BSA/PBS with 0.1% sodium azide.

11. Cut the wells and count in a gamma counter or expose the plate to film.

NOTES

i. Keeping the cells cold and in the presence of sodium azide throughout this procedure will slow the rate of capping and internalization of the surface antigens.

ii. In all of the antibody capture assays, the method for detecting the antibody can be substituted with other techniques. Most techniques employ either ^{125}I-labeled rabbit anti-mouse immunoglobulin antibodies or enzyme-linked rabbit anti-mouse immunoglobulin antibodies. Both these reagents can be purchased from commercial suppliers or prepared in the lab (p. 321).

ANTIBODY CAPTURE ON PERMEABILIZED CELLS—
CELL STAINING*

One major advantage of using cell staining in hybridoma screens is that the assays give an extra level of information. Unlike other antibody capture assays that rely on the simple detection of antibody, cell staining also determines the localization. This extra information makes cell staining a good assay when using complex antigens.

Both fluorochrome- and enzyme-labeled reagents can be used to detect the presence of the antibodies (see Chapter 10 for more details), but if the levels are high enough to be detected using enzyme-labeled reagents, enzyme methods should be used. Enzyme-labeled assays can be scored by using the light microscope. Scoring assays using the fluorescent microscope will give more resolution, but long-term observation under this microscope is disorienting for most people.

1. Grow cells in standard tissue culture conditions on standard tissue culture plates with fetal calf serum. Some staining will be more pronounced on subconfluent cells, some on fully confluent cells.

2. Pour off the medium, and flood the plate with PBS.

3. Pour off the PBS. Flood the plate with freshly prepared 50 : 50 acetone/methanol mixture. Incubate at room temperature for 5 min.

4. Pour off the acetone/methanol, and allow to air dry (approximately 5 min).

5. Score the bottom of the plate with a marking pen to form a grid of small squares to identify the location of the hybridoma tissue culture supernatants. Fifty tests can easily be done on one 100-mm tissue culture dish.

6. Add 2–5 μl of tissue culture supernatant to the fixed and permeabilized cell sheet above the appropriate mark. Incubate for 1 hr at room temperature in a humid atmosphere.

7. Wash the entire plate by flooding with PBS. Pour off the PBS and repeat three times.

8. For a 100-mm dish, add 3 ml of rabbit anti-mouse immunoglobulin antibody-horseradish peroxidase solution (diluted 1/200 in 3% BSA/PBS) to the plate. Incubate for 1 hr at room temperature.

9. Pour off and wash three times with PBS.

*Lane and Lane (1981); see also Chapter 10 for historical and alternative methods.

10. During the last wash, dissolve 6 mg of 3,3'-diaminobenzidine in 9 ml of 50 mM Tris (pH 7.6). A small precipitate may form. Add 1 ml of 0.3% (wt/vol) $NiCl_2$ or $CoCl_2$. Filter through Whatman No. 1 filter paper (or equivalent). This is sufficient for one 100-mm plate.

11. Add 10 μl of 30% H_2O_2. (H_2O_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.)

12. Add 10 ml of substrate solution per 100-mm dish. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 1–15 min.

13. Pour off the enzyme substrate and wash several times with water. Store in water with 0.02% sodium azide.

14. Look for black/brown spots in the marked squares and examine positives under the microscope.

NOTE

i. Cells grown in calf serum often show high background staining due to binding of the second antibody to calf immunoglobulins.

■ Antigen Capture Assays

In an antigen capture assay, the detection method identifies the presence of the antigen. Often this is done by labeling the antigen directly. These assays require the monoclonal antibody to have a high affinity for antigen since the labeled antigen is normally added at very low concentration in free solution.

There are two types of antigen capture assays, and these assays differ by the order in which the steps are performed. In one variation, the antibodies in the tissue culture supernatant are bound to a solid phase first, and then the antigen is allowed to react with the antibody. In the second variation, the antibody–antigen complex is allowed to form prior to the binding of the antibody to a solid phase. In either case, once the antibody–antigen complexes are bound to the solid support, the unbound antigen is removed by washing, and positives are identified by detecting the antigen.

Detection of the antigen can be performed by a number of techniques. If the antigen is available in pure form, it can be labeled by radiolabeling, fluorescent tagging, or enzyme coupling. If the antigen itself is an enzyme, positives may be determined by the presence of the enzymatic activity. Any property that is unique to the antigen can be used to identify positives.

ANTIGEN CAPTURE ON NITROCELLULOSE—
REVERSE DOT BLOT*

Reverse dot blot assays are more complicated to use than many of the other screening assays, but they are particularly valuable if pure or partially pure antigen is available, although only in limited quantities. The monoclonal antibodies in the supernatants are "captured" on an anti-immunoglobulin antibody layer, previously bound to nitrocellulose or PVC (p. 192 for this variation), and then labeled antigen is added. Positives can be determined by the location of the antigen. Because the antigen can be labeled to high specific activity with either ^{125}I or enzyme, very little antigen is used in the screening procedure. However, the assays are tricky to set up and demand careful use.

1. Prior to the assay, purify the immunoglobulin fraction from rabbit anti-mouse sera using one of the standard methods (p. 288). Purification on protein A beads is probably the easiest for the rabbit antibodies. Alternatively, purchase the purified antibodies from a commercial source.

2. Cut nitrocellulose paper to the size of a dot blot apparatus. Add rabbit anti-mouse immunoglobulin solution (approximately 200 μg of purified antibody/ml in PBS) to nitrocellulose paper. Use 10 ml/100 cm². Incubate for 60 min at room temperature.

3. Wash the paper three times with PBS, 5 min for each wash.

4. Incubate in 3% BSA/PBS with 0.02% sodium azide for 1 hr at room temperature.

5. Load into a 96-well dot blot apparatus.

6. Add 50 μl of hybridoma tissue culture supernatant to each well. Incubate for 1 hr at room temperature.

7. Draw the supernatant through the nitrocellulose paper by applying vacuum to the bottom chamber of the dot blot apparatus.

8. Wash the nitrocellulose paper and wells three times with 3% BSA/PBS.

9. Remove the paper from apparatus and incubate with ^{125}I-labeled antigen (10 ml/96-well sheet, 50,000 cpm/well in 3% BSA/PBS) at room temperature for 1 hr with shaking. (^{125}I-Labeled antigens can be prepared as described on p. 324.)

*Adapted from Wide and Porath (1966); Catt and Tregear (1967); Envgrall and Perlmann (1971); Van Weeman and Schuurs (1971).

10. Wash the paper with PBS until counts in the wash buffer approach background levels.

11. Cover in plastic wrap and expose to X-ray film at −70°C with a screen.

NOTES

i. The types of bonds that hold protein to nitrocellulose are not known. However, the binding is blocked by oils or other proteins. Wear gloves and use virgin nitrocellulose sheets. For short- or long-term storage, do not leave the paper in buffers containing other proteins. Slow exchange occurs and this will lower the strength of the signal. For long-term storage, freeze the paper in plastic wrap at −70°C.

ii. In all the assays in which proteins are bound to nitrocellulose, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

*ANTIGEN CAPTURE IN POLYVINYLCHLORIDE WELLS**

This assay is similar to the nitrocellulose reverse dot blot assay described on p. 190. The major difference is the use of polyvinylchloride (PVC) plates in place of nitrocellulose. This makes the handling of the individual assays easier, because each well is used for a separate assay; with the nitrocellulose, this is achieved by using a dot blot apparatus. The major disadvantage of using the PVC is the lower binding capacity of the PVC wells.

1. Add 50 μl of affinity-purified rabbit anti-mouse immunoglobulin in PBS (20 μg/ml) to each well. Incubate for 2 hr at room temperature or overnight at 4°C. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the PBS.

   Because of the low binding capacity of PVC, the signal will be stronger with affinity-purified rabbit anti-mouse immunoglobulin antibodies. These are the subset of antibodies in the anti-mouse immunoglobulin sera that bind to mouse antibodies. Affinity-purified antibodies can be prepared in the laboratory (p. 313) or can be purchased from commercial sources. When using concentrations above about 20 μg/ml, the solution should be saved for reuse.

2. Wash twice with PBS.

3. Add 200 μl of 3% BSA/PBS with 0.02% sodium azide to each well. Incubate for at least 2 hr at room temperature.

4. Wash twice with PBS. Add 50 μl of tissue culture supernatant to each well. Incubate for 2 hr at room temperature.

5. Wash three times with PBS. Add 50 μl of 3% BSA/PBS with 0.02% sodium azide containing 50,000 cpm of ^{125}I-labeled antigen per well. Incubate for 1 hr at room temperature. (Procedures for labeling antigens are described on p. 324.)

6. Wash with PBS until counts in the wash buffer approach background levels.

7. Either cut the wells apart and count in a gamma counter or expose the plate to X-ray film at −70°C with a screen.

*Adapted from Wide and Porath (1966); Catt and Tregear (1967); Envgrall and Perlmann (1971); Van Weeman and Schuurs (1971).

NOTE

i. In all the assays in which proteins are bound to polyvinylchloride the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ANTIGEN CAPTURE IN SOLUTION—IMMUNOPRECIPITATION

Immunoprecipitation is seldom used for screening hybridoma fusions, because the assays are tedious and time consuming. However, because the antigen is normally detected after SDS-polyacrylamide electrophoresis, it is simple to discriminate potential positives from authentic ones. The added information gained about the molecular weight of an antigen makes these assays particularly useful when using complex antigens.

1. Prepare sufficient radiolabeled antigen for overnight detection of 100 samples. Samples can be labeled directly using ^{125}I (p. 324) or prepared from radiolabeled extracts of cells (p. 429).

2. Either use tissue culture supernatants directly or pool in such a way that no more than 98 samples need to be handled (p. 215).

3. Label microfuge tubes and add samples of radiolabeled antigen to each. Add 50 μl of hybridoma tissue culture supernatants to each tube. Include one positive control (probably from the last test bleed) and one negative control (probably from a nonimmune mouse). Incubate for 1 hr on ice.

   If all classes of immunoglobulins are wanted, 30 min into the incubation add 0.5 μl of rabbit anti-mouse immunoglobulin serum. Normally it is easiest to add this as 10 μl of 1 in 20 dilution of the serum in PBS. Keep on ice.

4. Add 20 μl of a 10% suspension of prewashed SAC (p. 620). Incubate for 30 min on ice.

5. Spin for 1 min at 10,000g. Remove supernatant by aspiration. Resuspend the pellet in 750 μl of PBS.

6. Spin for 1 min at 10,000g and repeat wash.

7. Spin for 1 min at 10,000g and remove supernatant by aspiration. Add 50 μl of Laemmli sample buffer (p. 684). To make the resuspension easier, snap freeze by placing the tubes in a dry ice–ethanol bath. Resuspend and load onto a polyacrylamide gel. Handle as for normal electrophoresis (p. 636).

■ Functional Assays

In functional assays, the antibodies in the hybridoma tissue culture supernatants are used either to block a reaction or as a molecular handle to deplete an essential component of a reaction mix. Any antibodies that are identified using these assays form an immediately useful set of reagents. However, the assays are difficult to perform and interpret, and therefore are seldom used.

■ PRODUCING HYBRIDOMAS ▬▬▬▬▬▬▬▬▬▬▬▬▬▬

Although hybridoma production is the most discussed of the stages of monoclonal antibody preparation, most of the steps have been analyzed in enough detail that they are now routine. The ease with which this stage proceeds is dependent on how well the previous stages of immunization and development of the screen have gone. A strong immune response and the use of a good screening method will make the production of the hybridomas an easier task.

Once a good immune response has developed in an animal and an appropriate screening procedure has been developed, the construction of hybridomas is ready to begin. For the actual fusion, antibody-secreting cells are isolated from the appropriate lymphoid tissue, mixed with myeloma cells, centrifuged to generate good cell-to-cell contacts, and fused with polyethylene glycol (PEG). The fused cells are then removed from the PEG solution, diluted into selective medium, and plated in multiwell tissue culture dishes. Beginning approximately 1 week later, samples of the tissue culture supernatants are removed from wells that contain growing hybridomas and tested for the presence of the appropriate antibodies. Cells from positive wells are grown, single-cell cloned, and frozen. Finally, the monoclonal antibodies are collected and used.

Hybridoma production demands good tissue culture facilities and a worker with tissue culture experience. An experienced worker will be able to perform the entire fusion procedure from removal of the lymphoid tissue to the plating of the final fused cells in less than 2 hr. Little work is then required until the screening begins in about 1 week. This step is the most labor intensive of the entire project. Approximately 1 week is needed to complete the screening of the hybridoma wells, and if the fusion has been successful, another 2 weeks of tissue culture work will be needed until a suitable stage for a break has been reached. *Do not begin hybridoma production without the time needed for these operations.*

Although resultant hybridomas are relatively easy to grow, in the first stages following the fusion, they may be particularly fragile and need extra care. Because they are the final result of a long series of operations, and because they are produced as individual clones with no backup, the cells are quite valuable. At the early stages contaminated cultures cannot be recovered.

Chapter 7 (p. 245) contains descriptions of the techniques used for growing and maintaining hybridoma and myeloma cell lines as well as lists of appropriate growth media.

■ Preparation for Fusions

Prior to the time of fusion, several solutions must be prepared. In addition, unless you have purchased batches of fetal bovine serum and PEG that have been prescreened by manufacturers for their use in fusions, these solutions should be tested.

SCREENING FOR GOOD BATCHES OF FETAL
BOVINE SERUM

Only about one in five lots of fetal bovine serum (FBS) is particularly good at supporting hybridoma growth. The key constituents that distinguish good batches of serum from bad are not known. Order test batches from several suppliers or purchase prescreened serum directly from the distributor.

1. Test each batch of FBS against your present lot. Test the FBS with your most commonly used myeloma line as well as two hybridoma lines (if available). If possible, use one hybridoma that is easy to maintain and one that is more difficult.

2. For each lot of serum to test, prepare 30 ml of 10% FBS in medium (p. 247) without any further additives. Dispense 100 μl of the test medium in all the wells of a 96-well tissue culture dish using a multiwell pipettor. Prepare three trays per test and place the trays back in a CO_2 incubator to adjust the pH.

3. Wash the three test cell lines (one myeloma and two hybridomas) in medium without serum. The cells should be healthy and growing rapidly before the test. Resuspend the cells in medium without serum at a concentration of approximately 10^5 cells/ml. You will need approximately 2.5 ml for these tests.

4. Add 100 μl of the cell suspensions to each of the eight wells in the left-hand row of the test plates—one cell line per plate; three plates/sample of FBS.

5. Using an eight-well multipipettor mix the contents of the left hand row. Then remove 100 μl from the first row and do serial 1 in 2 dilutions across the plate. Incubate at 37°C in a CO_2 incubator.

6. Check the wells under a microscope after 7, 10, and 14 days. The wells in the left-hand side of the plate should all grow. Depending on the ability of the individual batches of serum to support growth, you will see growth extending to the wells with smaller number of cells. This assay tests directly for the ability of different serum samples to replace the feeder effects of high-density hybridoma culture and mimics the problems of individual cells attempting to grow out from either single-cell cloning or hybrid fusions.

NOTES

i. Good batches of serum should support growth of as few as 20 cells per well. Do not purchase batches that support less than 100.

ii. Any method that is used for single-cell cloning can be adapted to screen serum batches.

iii. Serum is stable when stored at −20°C for 1 year.

PREPARING OPI

OPI is a solution of oxaloacetate, pyruvate, and insulin that helps support the growth of hybridoma and myeloma cells at low densities. It is not required for high-density culture.

1. To prepare 100 ml of 100× OPI, dissolve 1.5 grams of oxaloacetate, 500 mg of sodium pyruvate in 100 ml of H_2O suitable for tissue culture work.

2. Add 2000 IU (international units) of bovine insulin.

3. Filter-sterilize.

4. Dispense in sterile tubes in 2.0-ml aliquots. Freeze at −20°C.

   OPI is stable at −20°C for 6 months to 1 yr.

PREPARING POLYETHYLENE GLYCOL

1. Melt PEG 1500 in a 50°C water bath. Place a small glass vial on a top-loading balance, and add melted PEG. Add either 0.5 gram or 0.3 gram of PEG, depending on which fusion method will be used (pp. 211 or 212). Most workers use PEG 1500 for fusions, but others use anything from PEG 1000 to PEG 6000 with good results.

2. Cap the vials and autoclave to sterilize.

PEG is stable at room temperature for many years.

NOTE

i. PEG can also be weighed dry and then autoclaved.

SCREENING FOR GOOD BATCHES OF POLYETHYLENE GLYCOL

Because fusions require so little polyethylene glycol (PEG), good batches will last a long time. Because differences in batches normally are small, and only the odd batch is unusable, most workers do not bother to test different lots of PEG. Bad batches contain trace amounts of toxic chemicals. Buy the highest grade of PEG that is available.

1. Add 100 μl of medium with 10% serum to each well of a 96-well microtiter dish (one plate per batch of PEG to be tested).

2. Wash myeloma cells by centrifugation at 400g for 5 min (approximately 10^6 cells per assay). Resuspend the cells in medium without serum and then respin.

3. While washing the myeloma cells, melt one vial (0.5 gram) of each of the PEG samples to be tested at 50°C (p. 201 for PEG preparation). Add 0.5 ml of medium without serum to each vial and place in a 37°C water bath.

4. Resuspend the cells in medium without serum and aliquot samples containing 10^6 cells into fresh centrifuge tubes (one tube per batch of PEG). Spin at 800g for 5 min. Carefully remove the supernatant from the cell pellet.

5. Resuspend each cell pellet with the 50% PEG solutions by pipetting. Include one control that is resuspended in medium without serum or PEG. Incubate at room temperature for 2 min. Add 10 ml of medium with 10% FBS. Take 100 μl of this suspension and dilute into a second 10 ml of medium with 10% FBS. Spin the final dilution at 400g for 5 min.

6. Aspirate the supernatant and resuspend the myeloma cells in 1.0 ml of medium with serum. Transfer 100 μl of the cell suspension into each of the eight wells on the left-hand side of the 96-well tissue culture dish. Using an eight-well multipipettor do 1 in 2 serial dilutions across the plate. Return to the CO_2 incubator.

7. Check the plates at day 14. Good batches of PEG will only slightly inhibit growth and will resemble the no PEG controls. Other batches should be discarded.

NOTE

i. The American Type Culture Collection (ATCC) and Boehringer Mannheim supply high-quality PEG that does not need to be tested prior to use.

■ Drug Selections

Hybridoma cell lines are selected by the addition of drugs that block the de novo synthesis of nucleotides (see p. 277 for details of the theory of drug selection). The most commonly used agents are aminopterin, methotrexate, and azaserine. All are effective agents to select against the growth of the myeloma fusion partner. When using aminopterin or methotrexate, de novo purine and pyrimidine synthesis are blocked, whereas azaserine blocks only purine biosynthesis. Consequently, aminopterin and methotrexate are supplemented with hypoxanthine and thymidine. Azaserine solutions are supplemented with hypoxanthine.

PREPARING HAT SELECTION MEDIUM*

Hypoxanthine, aminopterin, and thymidine selection (HAT) medium is commonly prepared from two stock solutions, 100× HT and 100× A.

1. To prepare 100 ml of 100× HT, dissolve 136 mg of hypoxanthine and 38 mg of thymidine in 100 ml of H_2O suitable for tissue culture. Heat gently (70°C) if they do not dissolve completely. The 100× stock is 10 mM hypoxanthine and 1.6 mM thymidine.

2. To prepare 100 ml of 100× A, add 1.76 mg of aminopterin to 100 ml of H_2O suitable for tissue culture. Add 0.5 ml of 1 N NaOH to dissolve. Titrate with 1 N HCl to neutral pH, being sure not to overshoot to acid pH, as aminopterin is sensitive to acid pH. The concentration of the 100× stock is 0.04 mM aminopterin.

3. Filter-sterilize the two solutions independently.

4. Dispense 2.0-ml aliquots in sterile tubes. Store at −20°C.

   Both 100× HT and 100× A are stable at −20°C for 1 year.

*Littlefield (1964).

PREPARING HMT SELECTION MEDIUM

HMT selection medium is commonly prepared from two stock solutions, 100× HT and 100× M.

1. To prepare 100 ml of 100× HT, dissolve 136 mg of hypoxanthine and 38 mg of thymidine in 100 ml of H_2O suitable for tissue culture. Heat gently (70°C) if they do not dissolve completely. The 100× stock is 10 mM hypoxanthine and 1.6 mM thymidine.

2. To prepare 100 ml of 100× M, add 49 mg of methotrexate to 100 ml of H_2O suitable for tissue culture. Add 0.5 ml of 1 N NaOH to dissolve. Titrate with 1 N HCl to neutral pH. The 100× stock solution is 1 mM methotrexate.

3. Sterilize the 100× HT and 100× M solutions by filtration.

4. Dispense 2.0-ml aliquots in sterile tubes. Store at −20°C.

   Both 100× HT and 100× M are stable at −20°C for 1 year.

PREPARING AH SELECTION MEDIUM*

1. To prepare 100 ml of 100× AH, add 0.136 gram of hypoxanthine in H_2O suitable for tissue culture. Heat to 70°C to dissolve, and then add 10 mg of azaserine. The 100× stock is 0.58 mM azaserine and 10 mM hypoxanthine. 100× H will be needed for growing hybridoma cells while removing the azaserine selection. 100× H is prepared as above, but without the addition of azaserine.

2. Filter sterilize.

3. Dispense into sterile tube in 2.0-ml aliquots. Store at −20°C.

   100× AH is stable when stored at −20°C for 1 year.

*Foung et al. (1982).

■ Final Boost

Three to five days before the fusion, the immunized mouse is given a final boost. This boost should be done at least 3 weeks after the previous injection. This interval will allow most of the circulating antibodies to be cleared from the blood stream by the mouse. Serum titers in the mouse begin dropping about 14 days after an immunization. If the levels of circulating antibodies are high, they will bind to the antigen and lower the effective strength of the boost.

The final boost is used for two purposes: to induce a good, strong response and to synchronize the maturation of the response. If this synchronization occurs, a large number of antigen-specific lymphocytes will be present in the local lymphoid tissue about 3 or 4 days after the boost. This will allow an increase in the relative concentration of the appropriate B-lymphocyte fusion partners. Consequently, the final boost should be directed to the source of the cell collection. In most cases, the spleen is the best choice for lymphocyte isolation, and, therefore, the final boost should try to localize the response to the spleen. This is best achieved by an iv injection done concurrently with an ip injection. If your source of antigen is limited, a single iv injection should be used. Remember that the antigen solution should be compatible with an iv boost (no Freund's adjuvant, SDS concentration below 0.1%, urea below 1 M, etc., see p. 110). If the antigen cannot be injected directly into the blood stream, an ip injection should be used, and the fusion should be done 5 days after the final boost.

In some specialized cases, for example foot pad injections or preparing IgA monoclonal antibodies, a regional lymph node may be the preferred site of lymphocyte collection. In the two examples given, the B-cell partners would be prepared from the inguinal node or from the Peyers patches, respectively.

■ Preparing the Parental Cells for Fusions

Prior to the fusion the myeloma cells that will serve as fusion partners must be removed from frozen stocks and grown. The technique below is designed to place the cells in the best conditions for the fusion, but any tissue culture techniques that keep them healthy and rapidly growing are suitable. On the day of the fusion, the antibody-secreting cells are isolated from the mouse. Both types of cells can be washed together just prior to the fusion (p. 210).

PREPARING MYELOMA CELLS FOR FUSIONS

Myeloma cells should be thawed from liquid nitrogen stocks at least 6 days prior to the fusion. Longer times may be necessary if the viability of frozen stocks is poor. The myeloma cells should be checked routinely for mycoplasma contamination, particularly if your laboratory or your tissue culture facility has a history of mycoplasma problems (p. 265). Any cells that test positive for mycoplasma should be replaced. The myelomas should be growing rapidly and healthy before the fusion. One day before the fusion, split the cells into fresh medium supplemented with 10% serum at a final concentration of 5×10^5 cells/ml (see p. 255 for methods for counting cells). On the morning of the fusion, dilute 10 ml of the overnight culture with an equal volume of medium supplemented with 20% FBS and 2× OPI.

Chapter 7 discusses the growth and maintenance of hybridoma cells (p. 245).

FIGURE 6.9
Splenectomy of a mouse.

PREPARING SPLENOCYTES FOR FUSIONS

If possible, at the time of the fusion you should collect the blood from the immunized animal to use as polyclonal sera specific for your immunogen. Collecting sera from laboratory animals is discussed in Chapter 5.

1. Sacrifice the mouse. See your local authorities on animal handling for advice on the proper humane procedures. Aseptically remove the spleen from an immunized animal (Fig. 6.9) and place in a 100-mm tissue culture dish containing 10 ml of medium without serum (prewarmed to 37°C). Trim off and discard any contaminating tissue from the spleen.

2. Tease apart the spleen using 19-gauge needles on 1.0-ml syringes. Continue to tease until most of the cells have been released and the spleen has been torn into very fine parts. Disrupt any cell clumps by pipetting. Transfer the cells and medium into a sterile centrifuge tube. As you transfer the cells, leave behind the larger pieces of tissue.

3. Wash the tissue culture plate and tissue clumps with 10-ml of medium without serum (prewarmed to 37°C) and combine with the first 10 ml in the tube.

4. Allow the cell suspension to sit for approximately 2 min. This will allow the larger cell clumps to settle to the bottom of the tube. Carefully remove the supernatant from the sediment and transfer to a fresh centrifuge tube.

   A spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

■ FUSIONS

Over the last 10 years a number of variations in fusion techniques have evolved. Most are based on the techniques of Galfre et al. (1977) or Gefter et al. (1977) and Kennett (1978). Examples of each are given below. Normally the fusion technique that is used does not make a major difference in the success of the fusion. You should use the method that is easiest and most convenient for you.

COMMENTS ■ Hybridoma Production

• Fusions are easy. If the immunization and the development of the screen have gone well, doing multiple fusions may be very helpful.

• If the drug selection you have chosen to use depends on thymidine in the culture media, mycoplasma contamination will doom the fusion. Mycoplasma are extremely efficient in converting thymidine to thymine (see p. 265 for methods of detecting mycoplasma).

• If the antigen is particularly valuable, it may be prudent to perform a practice fusion prior to using your reagents.

FUSION BY STIRRING (50% PEG)*

1. Wash the splenocytes twice by centrifugation at 400g in medium without serum (prewarmed to 37°C). At the second wash also spin 20 ml of the myeloma cells in a separate centrifuge tube. The myeloma cells should also be washed in medium without serum; they get one wash versus the two for the splenocytes. During these washes, melt a vial with 0.5 gram of PEG (p. 201) in a 50°C water bath. Add 0.5 ml of medium without serum and transfer the vial to a 37°C water bath.

2. After the final washes resuspend the two cell pellets in medium without serum (prewarmed to 37°C) and combine the cell suspensions. Centrifuge these cells together at 800g for 5 min. Carefully remove the medium as completely as possible.

3. Remove the 50% PEG solution from its container with a Pasteur pipet and slowly add the PEG to the cell pellet while resuspending the cells by stirring with the end of the pipet. Add the PEG slowly over 1 min. Continue stirring for an additional minute. Fill a 10-ml pipet with 10 ml of medium without serum (prewarmed to 37°C). Add 1.0 ml to the cell suspension over the next minute, while continuing to stir with the end of the 10-ml pipet. Then add the remaining 9.0 ml over the next 2 min with stirring. Centrifuge the cells at 400g for 5 min.

4. Remove the supernatant and resuspend the cells in 10 ml of medium supplemented with 20% prescreened fetal bovine serum (prewarmed to 37°C), 1× OPI, and 1× AH. Transfer the cells to 200 ml of medium with 20% prescreened fetal bovine serum (prewarmed to 37°C), 1× OPI, and 1× AH.

5. Dispense 100 μl of cells into the wells of 20 96-well microtiter plates using a multiple pipettor (a 96-well pipettor is easiest). Place at 37°C in a CO_2 incubator.

6. Clones should be visible by microscopy at about day 4 and by eye starting at about day 7 or 8.

NOTES

i. Other strategies can be employed for the number of wells into which the fusion is plated. These are discussed on p. 212. The one in the protocol above will yield approximately 2000 wells.

ii. Other drug selection methods can be used to select against the growth of the myeloma cells. These are described on p. 203. AH selection medium has only minor advantages over the other drug selections, but in general the hybridoma clones will appear more quickly in this medium. HAT selection is the most widely used.

*Galfre et al. (1977).

FUSION BY SPINNING (30% PEG)*

1. Melt a vial of 0.3 gram of PEG in a 50°C water bath. Add 0.7 ml of medium without serum and transfer to a 37°C water bath.

2. Centrifuge the spleen cells from the immunized animal at 400g for 5 min. At the same time centrifuge 20 ml of the myeloma cells. Resuspend both cell pellets in 5 ml of medium without serum.

3. Combine the two cell suspensions and transfer to a 15-ml round-bottomed centrifuge tube. Centrifuge for 5 min at 400g. Carefully remove all medium.

4. Add 0.2 ml of PEG solution. Suspend the cells by lightly tapping the tube.

5. Centrifuge for 5 min at 400g. Add 5 ml of medium without serum to disperse the pellet. Flick the tube, if necessary, to resuspend the cells. Do not pipet the cells. Then add 5 ml of medium with 20% fetal bovine serum (prescreened to support hybridoma growth, p. 198).

6. Centrifuge for 5 min at 400g. Remove the supernatant and re-suspend the cells in 10 ml of medium supplemented with 20% prescreened fetal bovine serum, 1× OPI, and 1× AH. Add the cells to 200 ml of medium supplemented with 20% prescreened fetal bovine serum, 1× OPI, and 1× AH.

*Adapted from Gefter et al. (1977); Kennett (1978).

Plating Strategies

The difficulty of the screen will determine the number of clones or pools of clones that can be tested in 1 day. This factor will determine the number of wells into which the fusion is plated. A number of plating strategies have been used successfully to identify positive hybridoma clones. There are no rules to govern the correct choice, but the suggestions given below may serve as a general guide.

1. If the screening procedure is easy (300 or more tests per day), dispense the fusion into a large number of wells in an attempt to have no more than one viable hybridoma per well (2000 or more wells). This is true whether the immunogen is very antigenic or not. The time needed in later stages of single-cell cloning will be reduced, and there is a greater chance that unique clones will not be lost by being overgrown by other cells prior to cloning.

2. If the screening procedure is moderately difficult (100-200 tests per day), the

7. Dispense 100 μl of cells into the wells of 20 96-well microtiter plate using a multiple pipettor (a 96-well pipettor is easiest). Place at 37°C in a CO_2 incubator.

8. Clones should be visible by microscopy at about day 4 and by eye starting at about day 7 or 8.

i. Other strategies can be employed for the number of wells into which the fusion is plated. These are discussed on p. 212. The one in the protocol above will yield approximately 2000 wells.

ii. Other drug selection methods can be used to select against the growth of the myeloma cells. These are described on p. 203. AH selection medium has only minor advantages over the other drug selections, but in general the hybridoma cells will appear more quickly in this medium. HAT selection is the most widely used.

number of wells to be used is determined by the strength of the immune response. If a strong response has developed, plate the fusion in a large number of wells (2000 or more wells). Because positive wells will arise at high frequency, even if all the wells do not get screened, a good number of positive clones can be identified. If the antigen has not elicited a strong response, and further immunizations have not improved this response, then either plate the fusion into 500 wells and test each well individually or plate into 2000 wells and screen pools of tissue culture supernatants. Determine the pool size by the total number of assays that can be performed in 1 day.

If the screening procedure is very difficult (50 or less tests per day), the number of wells to be used is determined by the strength of the immune response. If the immune response is good, plate the fusion into approximately 1000 wells and test pools of these cells. If the immune response is not strong, plate the fusion into 100 wells, and test the wells individually.

■ Feeding Hybridomas

Two strategies are used in deciding whether to feed hybridomas prior to screening. Early protocols suggested the removal of medium and then addition of fresh medium. This approach has not proven to be of much advantage, but does lead to more work. The addition of fresh medium to the hybridoma cultures at about day 4 or 5 after the fusion improves the general health of the cultures and will keep them rapidly growing throughout the screening procedure. Some workers prefer not to feed the cultures, allowing any poorly growing clones to die and then to concentrate on the hardiest of the clones that grow up. Feeding is done by adding 100 μl of fresh medium supplemented with 20% fetal bovine serum, 1× OPI, and 1× AH.

Pooling strategies

Pooling of tissue culture supernatants is an effective method to reduce the total number of tests that must be done in 1 day. The major difficulty encountered when supernatants are screened as pools is that the positive well in the pool will normally need to be identified by immediate re-screening.

In some cases the pool size may need to be adjusted during the screening. Also, for some assays the pool size will be limited by the sensitivity of the test. For example, some assays are dependent on the concentration of the positive antibody and dilution of the antibody during pooling may reduce the signal below the detection level.

Three types of pooling strategies are in common use.

Simple pools The most widely used method of pooling is a simple combination of several tissue culture supernatants. The most important variable is the choice in the number of wells that will form a pool. Because the goal of any screening strategy is to eliminate approximately 70% of the cultures, gauge the size of the pool so that only about 1 in 10 of the pools is positive.

Matrices If the positive supernatants are likely to be rare, arrange the tissue culture supernatants in a two-dimensional matrix. Because pools of supernatants are prepared from each vertical column and each horizontal row, the correct wells can be identified by the location of intersecting positives.

Soft agar An effective method of preparing pools of supernatants is to plate the original fusion in a semisolid medium that will allow the diffusion of antibodies, but will hold the hybridomas in place. This is most commonly done in soft agar (see p. 226 for the preparation of semi-solid media). After the soft agar has solidified, the cells are overlaid with regular tissue culture media. The antibodies can then diffuse into the liquid media and the media can be removed for testing. If a well is scored as positive, the clones within the agar are removed, grown up individually, and retested. One additional advantage of this technique is that the division rate of cells in soft agar is normally slower than in liquid media, so there may be extra time for screening. One disadvantage is that even the hardiest hybridomas do not have high plating efficiencies in soft agar and therefore the number of clones that arise in these conditions will be reduced.

Screening

Wells containing hybridomas are ready to start screening approximately 7–14 days after fusions (about day 7 for AH selection and days 10–14 for others). Representative wells at different times after fusion are shown in Figure 6.10. For most assays, clones that are just visible by eye are about the right stage for screening.

Some examples of appropriate screening methods are discussed on pp. 175–195. Other methods based on any of the techniques in Chapters 10–14 can be used as well.

1. When the clone or clones in a well are ready to screen, mark the wells with some convenient numbering system. Aseptically remove about 50 μl of tissue culture supernatant. This can be done conveniently either by using a pipettor or by removing the approximate amount with a Pasteur pipet. The supernatants are removed from the top of the medium without disturbing the hybridomas on the bottom. After removing the supernatant refeed the well with fresh medium.
2. Transfer the supernatant to a suitable container. If all the supernatant will be used in the screening assay, the hybridoma tissue culture medium can be transferred directly to the assay. However, it is normally more convenient to transfer the supernatants to a microtiter tray. Mark the tray to correspond to the original well number.
3. For most antigens, the supernatants are ready for testing without any special treatments. In some instances lower backgrounds can be achieved by removing any cells in the supernatants by centrifugation before testing.

Any remaining supernatant should be stored at −20°C. This can be used for confirmations, further testing, or identifying individual wells from pooled samples.

NOTE

i. Although splenocytes do not grow in standard tissue culture medium, they do not die immediately and will continue to secrete antibodies. This may lead to detecting false-positive wells in early screens. All positive cells should be rescreened prior to freezing or single-cell cloning.
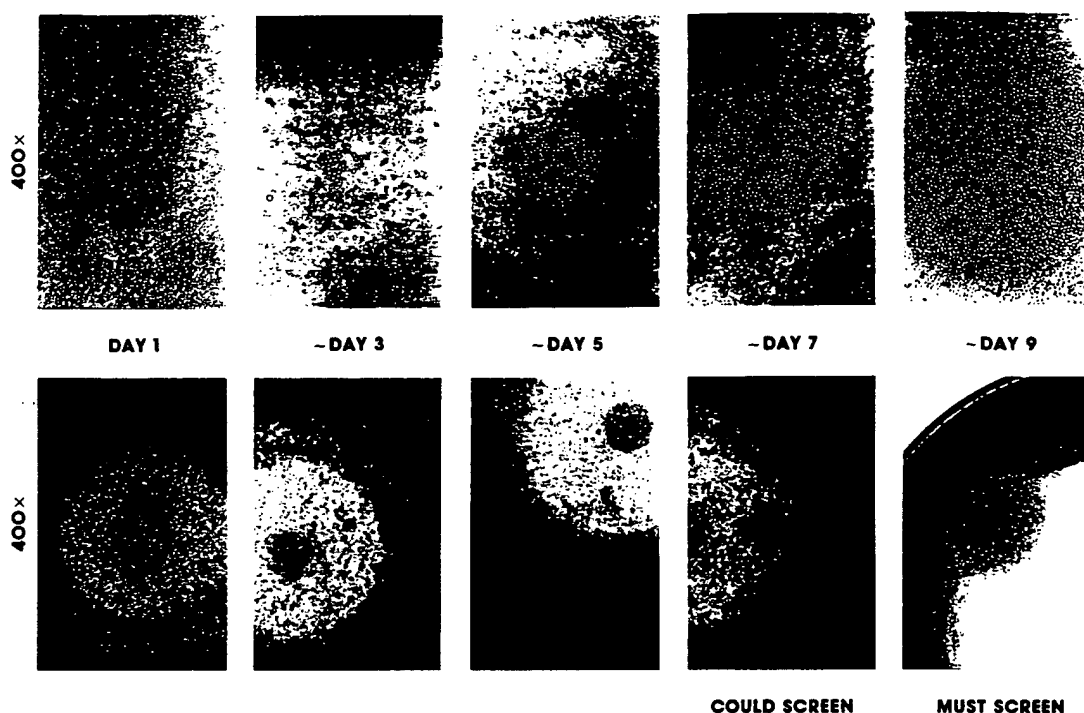
DAY 1   ~DAY 3   ~DAY 5   ~DAY 7   ~DAY 9

COULD SCREEN   MUST SCREEN

FIGURE 6.10
Representative wells at various days after fusion.

Detection Methods

Antibodies or antigens can be labeled with a number of compounds (see Chapter 9). Most common detection methods depend on reagents that are tagged with a radioactive isotope, a fluorescent compound, or an enzyme.

Radioactive isotopes. Any isotope that is easy to detect may be used to label antigens or antibodies. For example, proteins can be labeled in vitro by iodination or in vivo by growing cells in the presence of radioactive precursors. Radioactivity can be located by using β- or γ-counters or by normal X-ray film detection methods.

Fluorescence. Fluorochromes may be bound to either antibodies or antigens in vitro. Measurement of the bound fluorochrome will require an appropriate source of radiation and a detection device.

Enzymes. Antibodies and many antigens can be linked to functional enzymes in vitro. The antibody or antigen can then be detected by the addition of appropriate substrates for the enzyme. This is commonly achieved by using chromogenic substrates.

■ Expanding and Freezing Positive Clones

After a positive well has been identified, the cells are transferred from the culture in the 96-well plate to 0.5 ml of medium supplemented with 20% fetal bovine serum, 1× OPI, and 1× AH in a 24-well plate. After the 24-well culture becomes dense, it is transferred into 5.0 ml in a 60-mm dish and then to 10 ml in a 100-mm dish. Once the cells are transferred into the 60-mm dish, the drug selection can begin to be removed. This is done by first growing the cells for several passages in complete medium with hypoxanthine but lacking azaserine, or in complete medium with hypoxanthine and thymidine but lacking aminopterin or methotrexate. In either case, growing the cells with the base but without the drug allows all of the inhibitors to be diluted to a safe level before removing the bases.

At the 100-mm dish stage, the cells should be frozen. This is a convenient stage to collect 10 ml of supernatant, if any further testing of hybridomas needs to be done before concentrating on particular clones. However, if the correct clones have already been identified, the cells should be single-cell cloned as early as possible. This can be begun as early as at the 60-mm dish stage. Techniques for the freezing and storage of hybridoma cell lines are described on p. 257.

Often the transfer of hybridomas from one size of culture dish to the next is a difficult step to maintain cell viability. Presumably this is caused by the dilution of the growth factors in the medium and may be caused in part by overgrowth in the previous stage. If these problems exist, try using feeder cultures at these stages (see pp. 220–221 for the preparation of feeder cultures). Also, adding a sample of the diluted culture back into the original well will serve as a good backup if any problems arise.

COMMENTS ■ More Fusions?

After the screen has been completed, the decision on the appropriate next steps will depend on the number of positives that have been identified. If no positives are found, and the immunization yielded a strong response, the fusion should be repeated, but the choice of screening method should be reevaluated. If the immune response was weak, new approaches to the immunization should be tried. If only a few positive clones were identified, these should be tested as early as possible to determine whether they will perform adequately in the appropriate assays. If a comprehensive set of immunochemical reagents are needed, additional fusions are likely to be needed. If the fusion has been very successful (greater than 50 positives), it is seldom worthwhile and often practically impossible to carry and maintain all the clones. In these situations many of clones are likely to result from fusion of sibling antibody-secreting cells and therefore will not generate new antibody activities. Some sort of secondary screen should be considered to identify particularly valuable clones. This might be based on affinity or perhaps subclass of the resultant antibodies.

■ Single-cell Cloning

After a positive tissue culture supernatant has been identified, the next step is to clone the antibody-producing cell. The original positive well will often contain more than one clone of hybridoma cells, and many hybrid cells have an unstable assortment of chromosomes. Both of these problems may lead to the desired cells being outgrown by cells that are not producing the antibody of interest. Single-cell cloning ensures that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.

Isolating a stable clone of hybridoma cells that all secrete the correct antibody is the most time-consuming step in the production of hybridomas. Depending on the chances of the original positive being derived from a single cell, the easiest and quickest methods to prepare single-cell clones will differ. If the positive well contains multiple clones or if secretion of the antibody is highly unstable, the cloning should be done in two or more stages. In the first cloning, you should try to identify a positive well with only a few clones, and then try to isolate a single-cell clone from this stage. This often can be achieved by a combination of different cloning methods. For example, quick cloning by limiting dilution could be followed by cloning with a single cell pick.

Because hybridoma cells have a very low plating efficiency, single-cell cloning is normally done in the presence of feeder cells or conditioned medium. Good feeder cells should secrete the appropriate growth factors and should have properties that allow them to be selected against during the future growth of the hybridomas. Feeder cell cultures are normally prepared from splenocytes, macrophages, thymocytes, or fibroblasts.

> To ensure that a hybridoma is stable and single-cell cloned, continue repeating the cloning until every well tested is positive.

PREPARING SPLENOCYTE FEEDER CELL CULTURES

Although splenocyte feeders can be used immediately, they are most effective when they are prepared approximately 1 day before the single-cell cloning. Because spleen cells do not grow in normal tissue culture conditions, they are lost during the subsequent expansion of the hybridoma cells. Use a female mouse of the same genetic background as your hybridoma.

1. Sacrifice the mouse. See your local authorities on animal handling for advice on the proper humane procedures. Remove the spleen aseptically from the mouse and place in a 100-mm tissue culture dish containing 5 ml of medium without serum (see p. 209). Trim off and discard any contaminating tissue from the spleen.

2. Tease apart the spleen using 19-gauge needles on 1.0-ml syringes. Continue to tease until most of the cells have been released and the spleen has been torn into very fine parts. Disrupt any cell clumps by pipetting. Transfer the cells and medium into a conical centrifuge tube leaving behind all of the larger pieces of tissue. Wash these clumps and the plate with an additional 5 ml of medium without serum and combine with the first 5 ml.

3. Allow the cell suspension to sit at room temperature for approximately 2 min. This will allow the larger cell clumps to settle to the bottom of the tube. Carefully remove the medium and cells avoiding the sediment, and transfer to 100 ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth (1 spleen per 100 ml is about 10^8 cells/100 ml or 10^6/ml). Either use directly or prepare conditioned medium.

4. **Either:** To use directly for 96-well cloning (pp. 222–224), plate 50 μl of the spleen cell solution into each of the wells of a 96-well tissue culture dish. Allow to grow for 24 hr at 37°C.

   Or: To use directly for soft-agar cloning (p. 226), the medium with the feeder cells is used to dilute the hybridoma cell suspension prior to mixing with the soft-agar.

   Or: To prepare conditioned medium, transfer the splenocyte cell suspension to several tissue culture dishes. Place at 37°C in a CO_2 incubator for 3 days. Collect the cell suspension and remove the cells by centrifugation at 400g for 10 min. Filter sterilize and dispense in convenient sizes. Freeze at −70°C. Use the conditioned medium mixed 1:1 with medium supplemented with 20% FBS and 2× OPI.

NOTE

i. To avoid any possible problems with a particular spleen feeder culture, it may be best to combine several batches.

PREPARING FIBROBLAST FEEDER CELL CULTURES

Certain fibroblast cultures secrete the necessary factors to allow the growth of hybridoma cells at low plating densities. Early studies used fibroblast cultures that had been treated with mitomycin C or lethal doses of irradiation. Both of these treatments made it impossible for the feeder cells to contaminate future cultures of the hybridomas. More recently, this has been shown not to be necessary for fibroblast cultures that adhere strongly to the plastic tissue culture surface. Other studies have compared the ability of different fibroblast cells to support single-cell cultures of hyridoma cells and have found that the human diploid cells MRC 5 are the most effective in this test. These cells are not an established cell line, and so will need to be replaced in the future by another source. The MRC 5 cells are currently available from several sources including the American Type Culture Collection.

1. The MRC 5 cells are grown and maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. They should be used at passages below 40.

2. Trypsinize the cells and count. Prepare a solution of 2×10^5 cells/ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth.

3. For cloning using 96-well tissue culture dishes (pp. 222–224), add 50 μl of the cell suspension to the wells. Allow the cultures to grow for 1 day at 37°C.

   Or: For soft-agar cloning using 60- or 100-mm tissue culture dishes (p. 226), add 10 ml of the cell suspension to a 100-mm dish or 3 ml to a 60-mm dish. Allow the cells to adhere to the plastic overnight at 37°C. Remove the medium and add the soft agar hybridoma cell suspension to the plate.

SINGLE-CELL CLONING BY LIMITING DILUTION

Cloning hybridoma cells by limiting dilution is the easiest of the single-cell cloning techniques. Two approaches are given below, one rapid technique for generating cultures that are close to being single-cell cloned and one for single-cell cloning directly.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, limiting dilution cloning should be done at least twice to generate a clonal population.

Limiting Dilution (Rapid)

1. Using a multiwell pipettor (8-, 12-, or 96-well), add 50 μl of medium with 20% FBS and 2× OPI to each well of a 96-well plate. The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 100 μl total volume/well.

2. The hybridomas should be growing rapidly. Remove 100 μl of the hybridoma cell suspension using a pipetman and transfer to the top left-hand well. Mix by pipetting.

3. Do 1 in 2 doubling dilutions down the left-hand row of the plate (8 wells, 7 dilution steps). Discard tip.

4. Do 1 in 2 doubling dilutions across the plate using an 8-well multi-pipetter.

5. Clones should be visible by microscopy after a few days and normally will be ready to screen after 7–10 days. Score the wells by microscopy. There should be a line running on a 45° diagonal that contains approximately the same number of clones per well. If the cells are nearly cloned when you start, screen only wells with one or two clones. If not, screen a selection of wells with multiple clones as well as all those with only one clone.

6. Select the best wells and either grow up or repeat the cloning procedure directly.

Limiting Dilution (Slow)

1. The hybridomas should be healthy and rapidly growing at the time of cloning. Prepare four dilution tubes with medium supplemented with 20% fetal bovine serum and 2× OPI for each cell to be cloned. Three tubes should have 2.7 ml and the fourth should have 3.0 ml.

2. Add 10 μl of the hybridoma cells to the tube containing the 3.0 ml of medium. Do 1 in 10 dilutions of the hybridomas by removing 0.3 ml and transferring into the 2.7-ml tubes.

3. Add 100 μl of each dilution into 24 of the wells of a 96-well tissue culture plate (24 wells/dilution; 4 dilutions/plate, i.e., one hybridoma/plate). The wells should already contain 50 μl of feeder cells (pp. 220 or 221), giving 150 μl total volume/well. If the cells from the highest dilution are plated first, then the pipet does not need to be changed during the plating.

   If many hybridomas are being cloned at the same time, it may be worthwhile to plate the dilutions by using a 10-ml or larger pipet. One drop from these pipets will deliver approximately 100 μl.

4. Clones will begin to appear in 4 days and should be ready to screen starting about days 7–10.

   Screens can be done from wells containing multiple clones as well as from wells containing only single clones.

SINGLE-CELL CLONING BY PICKS*

Cloning hybridomas by picking a single cell from a growing culture is the only cloning method that ensures that clones arise from a single cell. During the cloning procedure, the cell is followed under the microscope to be certain that the clone comes from only one cell.

1. Add approximately 100 μl of medium with 20% FBS and 2× OPI to the wells of a 96-well plate (approximately 20 wells/hybrid). The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 150 μl total volume/well.

2. At the time of the cloning all cells should be growing rapidly. Do serial 1 in 5 dilutions of the hybridoma cells in 60-mm dishes. Use about 0.3 ml into 1.2 ml; this will allow enough volume to cover the bottom of the plate, but not so deep as to make the pipetting difficult. Observe the cells under the microscope and choose a plate with well-separated cells.

3. Use a drawn out 50-μl capillary pipet connected to a mouth pipetting device with a 0.2-μm filter fitted in the line. Partially fill the pipet with complete medium from a separate plate without cells. While watching under the microscope, draw a single cell into the pipet. Move to an area of the plate without any cells and blow out the cell to make sure you have only one cell. Draw it up again and transfer to one of the wells with feeders. With practice, single-cell picks take about 1 min.

4. The clones should be ready to screen in 7–10 days.

*J. Wyke (pers. comm.).

NOTE

i. Because this technique demands working under the microscope on the open bench, one might expect contamination to be common. However, the only portion of the tissue culture medium that is exposed to the open air for long is the dish that you are picking from and you only transfer a very small volume at one time. So the chances of contamination are low. Needless to say, this technique should only be done in an area without drafts.

SINGLE-CELL CLONING BY GROWTH IN SOFT AGAR

Cloning of hybridoma cells in semisolid medium is one of the most commonly used methods for producing single-cell clones. The technique is easy, but, because it is performed in two stages, it does take longer than other methods. Not all cells will grow in soft agar, and there may be a bias on the type of colony that appears. However, most of the commonly used myeloma fusion partners have relatively good cloning efficiencies in soft agar, and consequently, so do most hybridomas.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, single-cell cloning in soft agar should be repeated at least twice before the cells are considered clonal.

1. Prior to cloning prepare 3% agarose (Seaprep 15/45, FML Corporation or equivalent) in H_2O suitable for tissue culture. Sterilize by autoclaving. This is stable for 6 months to 1 year.

   Prepare double-strength medium, normally from powdered medium. Add 100 μg/ml of gentamicin, and sterilize by filtration. Store at 4°C. Stable for about 1 month at 4°C, but at that time if fresh glutamine is added to 2 mM the shelf life can be extended to 3 months.

2. Melt agarose in a boiling water bath or in a microwave oven and cool to 37°C.

3. To the 2× medium add fetal bovine serum to 20% and OPI to 2×. Warm to 37°C in a water bath.

4. Cells should be healthy and growing rapidly at the time of cloning. The cells should be as free of clumps as possible. Do 1 in 10 dilutions of hybridomas in 1× medium. If not using feeders, the 1× medium is prepared by diluting a sample of the complete 2× medium with sterile H_2O. If using feeders that grow in suspension, the medium used for these dilutions should be the cell suspension from the feeder cell preparation (p. 220). If using fibroblast feeders (p. 221), these cells should be plated on the tissue culture dishes to be used for the cloning 24 hr earlier, and the 1× medium should be prepared by diluting the 2× complete medium.

5. Add 150 μl of cells from the dilutions between 10^5 and 10^2 cells/ml to 60-mm tissue culture plates (2 plates/dilution). Do not bother to count cells. If you are uncertain about the exact concentration of cells, it is easier to do an extra dilution than to count the cells.

6. Mix the 3% agarose and the 2× medium 1 : 1. Add 4 ml to each plate, and mix by pipetting.

7. Place the plates at 4°C for 45 min and then transfer to 37°C in a CO_2 incubator.

8. Macroscopic clones will appear beginning about day 10. Pick clones from the highest dilution that shows growth. Remove a plug of agarose containing the colony with a sterile Pasteur pipet. Transfer the plug to 1 ml of medium in a 24-well plate. Disperse the clone by pipetting.

9. Supernatants from these wells will normally be ready for screening 48–72 hr later.

NOTE

i. As an alternative, the cells may be grown in the dilution tubes themselves (Civin and Banquerigo 1983). Add 2 ml of the 1.5% agarose/medium solution to each tube and grow as described above.

■ Unstable Lines

If hybridomas continue to produce less than 100% positive wells, even after four or more single-cell cloning steps, the lines probably have an unstable assortment of chromosomes. If the antibodies produced by these cells are particularly valuable, extra work to save these lines may be necessary. Two strategies are used. In the first and most straightforward, the single-cell cloning is continued on a regular basis, trying to isolate a stable subclone. Perhaps surprisingly, this often works. The screening assays should be adjusted to screen not only for the presence of the appropriate antibody, but also for the levels of antibody produced. Wells that contain a stable subclone of the original should produce higher levels of antibodies. If the stable variant is generated early in the proliferation within a well, the differences in antibody production between the well containing the variant and those that do not will be significant. At this stage many workers stop screening with an antigen-specific assay and only screen for the level of mouse antibody produced (see p. 560 for examples). After a stable line is generated, the specificity of the antibody should be reestablished.

A second strategy is to refuse the important line with a myeloma and allow the chromosomes to reassort from the beginning, hoping to isolate the stable variant from this source. To date, most re-fusions have been done by standard techniques and extensive screening. However, the introduction of a selectable drug selection marker into a suitable myeloma cell line should make selection against the parental myelomas easier. The hybridoma would carry a functional HPRT gene, while the myeloma would carry, for example, a neomycin gene. Selection for both genes should yield only successful secondary hybridomas.

■ Contamination

During the early stages of the fusion, contamination will mean the loss of the well or the fusion; however in later stages, important hybridomas can sometimes be saved.

CONTAMINATION IN THE FUSION WELLS—
A FEW WELLS ONLY

1. Contaminated wells can be identified by their unusual pH or turbidity. Confirm the presence of the contaminating organisms by observing under the microscope. Mark the wells.

2. Move to the tissue culture hood and carefully remove the lid. If the underside of the lid is damp, replace with a new lid. Dry the top and edges of the plate itself by aspiration before replacing. If there is contaminated medium on the lid, autoclave the whole plate without any further work.

3. Remove the medium from the contaminated well by aspiration. Try to avoid generating any aerosols. Add enough 10% bleach to the well to bring the level right to the rim. Allow it to sit for 2 min at room temperature.

4. Remove the bleach from the contaminated well by aspiration. Add enough ethanol to the well to bring the level right to the rim. Remove by aspiration and repeat.

5. Dry the well by aspiration.

CONTAMINATION IN THE FUSION WELLS—GROSS

1. Autoclave the plates.

CONTAMINATION OF A CLONED LINE

1. If the line has been frozen, it is easiest to go back to the most recent freeze down and thaw a fresh vial of the cells.

2. If the line has not been frozen, inject the cells into mice that have been primed for ascites production (p. 274). The animals must be of a compatible genetic background to your hybrids (e.g., BALB/c × BALB/c into BALB/c or BALB/c × C57Bl/B6 into BALB/c × C57B1/B6 F_1). If no mice have been primed with 0.5 ml of pristane the required 1 week in advance, inject 0.5 ml of Freund's adjuvant into the peritoneum. Wait 4 hr to 1 day and inject the hybridomas. Inject at least two mice for each contaminated culture.

3. When and if ascites develop, tap the fluid and transfer into a sterile centrifuge tube (see p. 274 for more information on ascites production).

4. Spin the ascites at 400g for 5 min at room temperature.

5. Remove the supernatant. Resuspend the cell pellet in 10 ml of medium supplemented with 10% fetal bovine serum and transfer to a tissue culture plate. The supernatant can be checked for production of the appropriate antibody. If positive, save for use.

6. Handle as for normal hybridomas, except keep the cells separate from the other cultures until there is little chance of the contamination reappearing.

The success rate may be as high as 80%.

NOTE

i. Animals injected with infected cultures should be kept isolated from the main animal colony.

■ Classing and Subclassing of Monoclonal Antibodies

Many techniques for using monoclonal antibodies require antibodies with specific properties. One set of these properties is unique to the individual antibody itself and includes such variables as specificity and affinity for the antigen. These properties all depend on differences in the antigen-combining domain of the antibody and can be assayed by comparing the properties of the monoclonal antibodies in tests that measure antigen binding activity.

A second set of important properties for monoclonal antibodies is determined by the structure of the remainder of the antibody, sequences encoded by the antibody common regions. These properties include the class or subclass of the heavy chain or the light chain. The different classes or subclasses will determine the affinity for important secondary reagents such as protein A (see p. 616). The type of heavy and light chain can be distinguished by simple immunochemical assays that measure the presence of the individual light- and heavy-chain polypeptides. This is normally achieved by raising antibodies specific for the different mouse heavy- and light-chain polypeptides (p. 622). The production of these antibodies is possible because the light- and heavy-chain polypeptides from different species are sufficiently different to allow them to be recognized as foreign antigens. Most often these anti-mouse immunoglobulin antibodies are raised in rabbits as polyclonal sera, and then the antibodies specific for a particular heavy or light chain are purified on immunoaffinity and immunodepletion columns. Although these chain-specific rabbit anti-mouse immunoglobulin antibodies can be made in the laboratory, it is normally easier to purchase them from commercial sources. There are a large number of different assays used, and some of the more common are listed below.

DETERMINING THE CLASS AND SUBCLASS OF A MONOCLONAL ANTIBODY BY OUCHTERLONY DOUBLE-DIFFUSION ASSAYS*

Originally, the Ouchterlony double-diffusion assays were the most common method for determining class and subclass of a monoclonal antibody. They have been largely superseded by other techniques, but they still are useful, particularly when only a few assays will be performed. In these assays, samples of tissue culture supernatants (often concentrated tenfold) are pipetted into a well in a bed of agar. Class- and subclass-specific antisera are placed in other wells at equal distance from the test antibody. The two groups of antibodies diffuse into the agar. As they meet, immune complexes form, yielding increasing larger complexes as more antibodies combine. When large multimeric complexes form, the immune complexes will precipitate, forming a line of proteins that is either visible to the naked eye or that can be stained to increase the sensitivity. The precipitated proteins form what is referred to as a precipitin line.

1. Prepare a 10-ml sample of tissue culture supernatant from a hybridoma. Grow the cells in medium supplemented with 10% FBS and allow the culture to overgrow and die.

2. Spin the tissue culture supernatant at 1000g for 10 min. Collect the supernatant. If the supernatant is not clear of all debris, either filter it through a 0.45-μm filter (sterility is not important) or spin at 7000g for 15 min.

3. Concentrate the supernatant 10-fold using an ultrafiltration manifold. This is most easily done with adaptors that are designed to concentrate in the centrifuge. Many of the ultrafiltration specialty companies now supply these devices; follow the manufacturers' instructions. Remove the tissue culture supernatant when the 10 ml sample has been reduced to 1 ml.

   Tissue culture supernatants may also be concentrated by ammonium sulfate precipitation (p. 298).

4. Prepare a 1.4% agarose solution in PBS with 5 mM EDTA. Melt the agarose in a boiling water bath or in a microwave. Cool to 45°C.

   Ouchterlony plates can also be purchased commercially.

5. On a level surface pipet 3 ml of the agarose solution onto the top of a 3 × 5-cm clean glass slide. The agarose should form a layer about 2 mM deep. The surface tension of the agarose should hold the agarose on the slide. Allow to harden at room temperature.

*Ouchterlony (1949).

6. Using a 200-μl capillary pipet or a commercial apparatus, careful-ly core vertical small holes in the agarose in a pattern that looks like this:
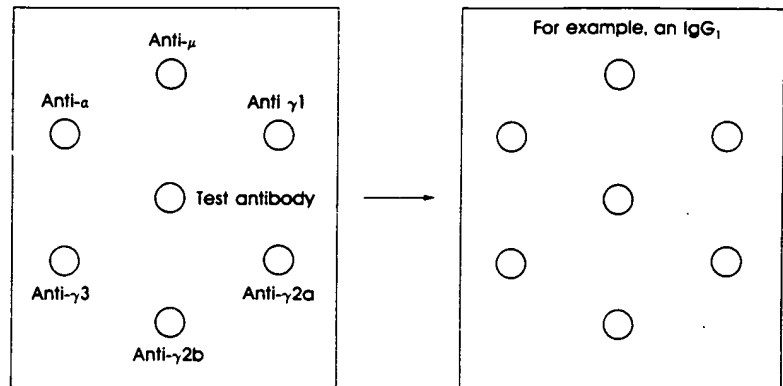
If the capillary pipet is attached to a pipetting device, a light suction while preparing the wells will allow the plugs to be with-drawn easily.

7. Add 5 μl of rabbit anti-mouse immunoglobulin sera specific for the various classes, subclasses, or light chains to each of the wells in the outer ring.

8. Add 5 μl of the concentrated tissue culture supernatant to the middle well.

9. Incubate in a humid atmosphere overnight at room temperature.

.10. Score positive reactions by the appearance of a precipitin line between the wells with reactive antibodies.

NOTE

i. The sensitivity of these assays can be increased by staining the bands with Coomassie brilliant blue. Cover the gel with wet filter paper and place in a 50°C oven. Incubate until dry. Wet the paper and remove from the gel. Wash for 30 min in several changes of PBS. Repeat the drying procedure. Stain with Coomassie for 15 min (p. 649). Destain in 7% acetic acid, 25% methanol.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTIGEN-COATED PLATES

Any of the assays used to screen hybridoma fusions that detect antibodies with a secondary anti-mouse immunoglobulin antibody can be adapted to screen for class or subclass. For example, if the detection method used ^{125}I-labeled rabbit anti-mouse immunoglobulin to locate antibodies bound to the antigen, then substituting anti-class or subclass-specific antibodies for the ^{125}I-reagent will identify the type of heavy chains. An example of these types of reactions is given below using an antigen bound to 96-well PVC plates, but similar tests could be developed for any of the antibody capture assays.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

5. Fill the wells with 3% BSA/PBS (no sodium azide). Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of each tissue culture supernatant to be tested to every well of a vertical row (8 wells/test). Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS (without sodium azide) containing a dilution of horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin class- or subclass-specific antibody to each well as shown below:

TEST ANTIBODIES

Incubate 1 hr at room temperature. (Horseradish peroxidase-labeled reagents can be purchased or prepared as described on p. 344. Most commercial reagents should be diluted 1 in 1000 to 1 in 5000. Try several dilutions in preliminary tests and choose the best.)

10. Wash the plate with PBS three times.

11. During the final washes prepare the TMB substrate solution. Dissolve 0.1 mg of 3',3',5',5'-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is sufficient for two 96-well plates. (Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.)

12. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

13. Incubate for 10–30 min at room temperature. Positives appear pale blue.

14. Add 50 μl of stop solution, 1 M H_2SO_4, to every well. Positives now appear bright yellow. To quantitate the binding, read the results at 450 nm.

NOTE

i. Do not include sodium azide in solutions when horseradish peroxidase is used for detection.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTI-Ig ANTIBODIES

One of the easiest methods for determining the class and subclass of a monoclonal antibody is to bind class- or subclass-specific antibodies to the wells of a polyvinylchloride (PVC) plate. The test monoclonal antibody is added to each well, but will bind only to wells coated with antibodies that are specific for its subclass or class. These bound antibodies are detected using a secondary antibody specific for all mouse antibodies.

1. Purify the antibodies from rabbit anti-mouse immunoglobulin class- or subclass-specific antibodies. Techniques for these purifications are discussed in Chapter 8. For most purposes, protein A beads are probably the easiest to use. (Rabbit anti-mouse immunoglobulin class- and subclass-specific sera can be purchased from several suppliers.)

2. After purification dilute the antibodies to 20 μg/ml in PBS. Add 50 μl to the wells of a PVC plate in the pattern below. Each monoclonal antibody being tested will need one row.
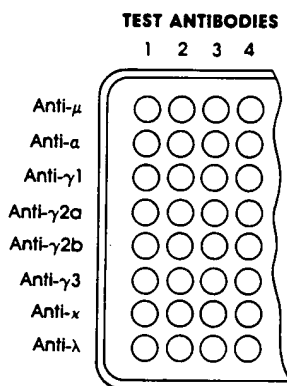
3. Incubate for 2 hr to overnight at room temperature in a humid atmostphere.

4. Remove the antibodies and save for future use. The antibodies can be reused approximately five times.

5. Fill the wells with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr or overnight at room temperature.

6. Wash three times with PBS. Add 50 μl of tissue culture supernatant from each hybridoma to the appropriate wells.

7. Incubate at room temperature for 2 hr in a humid atmosphere. Shake out the unbound antibody, and wash three times with PBS.

8. Add 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin antibody to each well (diluted in 3% BSA/PBS with 0.02% sodium azide).

9. Incubate for 2 hr at room temperature in a humid atmosphere. Discard the iodinated antibodies in an appropriate waste container.

10. Wash the wells three times with PBS. Cut the wells from the plate and count in a gamma-counter.

NOTE

i. Other detection methods can be substituted for the iodinated antibodies. Common alternatives include enzyme-labeled reagents.

Hybridoma cells should be washed by centrifugation. Resuspend the cell pellet in medium supplemented with 20% fetal bovine serum at a density of 10^4 cells/ml and dispense 100 μl into the wells of 10 96-well microtiter plates. This yields approximately 1000 cells per well with about 1000 wells. Therefore, about 10^6 cells are being screened per assay. After the cells have grown, remove a sample of the tissue culture supernatant and screen for the presence of the IgG or IgG_{2a} antibodies. Between one and five positive wells may be seen. Choose the strongest positive, and transfer these cells to fresh medium. Continue passaging the cells until they are numerous enough to clone again. In the second round, the cells should be plated at 100 cells per well. The procedure is repeated and then the cells are plated at 10 cells per well. In the last round the cells are single-cell cloned using one of the techniques described on p. 219.

■ INTERSPECIES HYBRIDOMAS

Antibody-secreting cells isolated from one species but fused with myelomas from another species yield interspecies hybridomas. These types of fusions were common in the early years of hybridoma production. Often these hybrids would be formed by immunizing rats and fusing with mouse myeloma cells. This was done before good rat myeloma fusion partners were available. These fusions yield hybridomas that secrete rat antibodies, but the hybridoma cells cannot be grown conveniently as ascites tumors. Therefore, antibody production is almost entirely limited to tissue culture sources.

Although some important monoclonal antibodies have been produced using interspecies fusions, there seems little need for using these types of fusions today.

■ HUMAN HYBRIDOMAS

One of the most exciting areas for hybridoma research over the last 5 years has been the development of systems for the production of human hybridomas. Human monoclonal antibodies will be used extensively for clinical applications. Although this field has been marked by exciting publications announcing new breakthroughs, the actual progress in setting up the routine production of human hybridomas for laboratory use has been slow. For most research applications, producing human hybridomas still does not offer many, if any, advantages. The two most successful strategies that are used are standard fusions with human myeloma cells and the use of the Epstein-Barr virus (EBV) to transform antibody-secreting cells. One of the major problems in producing human hybridomas has been the lack of a suitable myeloma partner. Several of these lines have been isolated and are now in use.

The use of EBV-transformation to allow antibody-secreting cells to grow in standard tissue culture systems has solved some of the problems in human monoclonal antibody production. One unfortunate drawback of this approach is that the resultant transformants seldom secrete large amounts of antibodies. This has been overcome in some cases by fusing the EBV-transformed cell with a mouse myeloma cell line to allow the secretion of large amounts of antibodies. The combined use of EBV and secondary fusions points out two important aspects in hybridoma research. One is the use of other vectors to deliver important genetic information such as oncogenes. Second, if a particular hybrid does not possess all of the properties that are needed for a particular use, the line may be refused with other hybrids to achieve these properties.

There are several publications that describe progress in the isolation of human antibody-secreting cells, and these types of references should be checked for the details of producing human hybridomas.

■ FUTURE TRENDS ■■■■■■■■■■■■■■■■■■■■■■■■■■■

Few changes in the techniques used to produce hybridomas have been adopted since the original methods of Köhler and Milstein were reported. However, hybridoma construction is likely to change radically during the next 10 years. In several areas, preliminary work has already been reported that will form the basis for more widespread use of new techniques.

1. **In vitro immunizations** Although the first in vitro immunization procedures were described in the early 1980s, they have not come into common use. The two major advantages of in vitro immunizations are the small amount of antigen that is required (as low as 1 ng) and the lack of cellular regulation on the developing immune response. Both of these factors make in vitro immunizations a potentially powerful technology. They have not been widely used to date, because so far they do not allow the development of high-affinity antibodies and because many of the antibodies that are produced are from the IgM class.

2. **Electrofusion** PEG fusions routinely produce one viable hybridoma from 10^5 starting cells, and this may be below the needed efficiency. One method that is gaining more widespread use is fusing cells by applying high-voltage electrical gradients across cell populations—short bursts fuse adjacent membranes and yield hybrid cells. This method has been applied successfully to hybridoma production, and the higher fusion efficiency allows production of more hybrid cells. In general, this has not been important for most fusions, because hybridoma production is normally limited by the screening method rather than by the frequency of hybridoma production. As more rapid screening procedures are developed, this fusion method will become more important. Also, as techniques are developed that allow the selection of the desired antibody-secreting cell prior to fusion, this and other high-efficiency methods will become increasingly valuable.

3. **Retroviral vectors** Recombinant retroviral vectors hold the most promise for the efficient transformation of antibody-secreting cells. These vector systems can be engineered to deliver oncogenes into cells. However, the exact gene or combination of genes that will immortalize plasma cells but will not affect antibody secretion has not been determined. Also, because there will be little discrimination between the desired parental cells and undesired ones, this technology will be useful only when other methods of physically isolating the correct antibody-secreting cell are routinely used.

4. **Antigen-directed fusions** A number of methods are being developed that, prior to fusion, physically couple myeloma cells with cells that are secreting the desired antibodies. These techniques take advantage of the antigen-combining site of surface antibodies found on some secreting cells. This combining site is used as a target for a modified antigen that will also bind to myeloma cells. After fusion, the frequency of appearance of hybridomas secreting the desired antibodies is much higher than in undirected fusions.

5. **Fusion partners** More sophisticated methods of identifying cells that secrete the desired antibodies are being developed. Most of these methods use a fluorescence-activated cell sorter to identify and purify cells with surface immunoglobulins having the correct specificity. These cells then can either be fused with myeloma cells or transformed by other methods. These technologies will continue to improve, giving better and more refined choices for antibody selection prior to fusion or transformation. In addition, new myeloma fusion partners are constantly being described that have better properties for successful fusions.

6. **Defined medium** Many of the growth factors that are necessary for the cultivation of hybridomas have been identified, and several defined medium have been developed. These culture conditions allow hybridomas to be grown in medium that do not contain other immunoglobulins (often bovine), and the low levels of proteins in these solutions make purification of antibodies from the tissue culture supernatants easier.

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Contents

Monoclonal Antibodies to a New Antigenic Marker in Epithelial Prostatic Cells and Serum of Prostatic Cancer Patients

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ABSTRACT

Stable clones of murine hybridomas 7E11-C5 and 9H10-A4 were obtained following immunization with LNCaP cells. The LNCaP cells were isolated from a human prostatic cancer (Ca)). Both hybridomas secreted monoclonal antibodies (MoAb) of the IgG1 subclass which were reactive with the insoluble, cytoplasmic, membrane rich fractions of the immunogen. Neither MoAb reacted with the soluble cytosol of LNCaP cells nor with purified human prostatic acid phosphatase (PAP) nor prostate specific antigen (PSA). MoAb 9H10-A4 reactivity was very narrow and limited to the surfaces of LNCaP cells only. MoAb 7E11-C5 specificity was restricted to human prostatic epithelium, both normal and malignant. Except LNCaP, none of the 32 lines of human normal or neoplastic cells reacted with MoAb 7E11-C5. In a survey of frozen sections from 175 human specimens, positive indirect immunoperoxidase staining was limited to epithelium in all 11 specimens of localized and metastatic CaP, 7 benign prostatic hypertrophy (BPH) cases and 7 normal prostates. None of the 26 various nonprostatic tumors nor 120 out of 122 specimens from 28 different normal organs were reactive. Positive staining occurred in 2 out of 14 normal kidneys. Competitive binding with MoAb 7E11-C5 or its F(ab')$_2$ fragments demonstrated the presence of circulating epitope 7E11-C5 in 20 out of 43 sera from CaP patients. Only 3 out of 66 sera from nonprostatic malignancies reacted. None of 30 normal blood donors sera nor 7 BPH sera were positive. Thus, highly significant ($p<0.0001$) association between diagnosed prostatic cancer and circulating molecules expressing the epitope reactive with MoAb 7E11-C5 was established. Significant probability ($p<0.05$) also suggested that patients with positive ELISA test are more likely to be in progression, than those who are negative. These results suggest that this apparently new antigenic marker may be of clinical potential in CaP.

INTRODUCTION

In 1978, we established in vitro the LNCaP cell line (1,2) from a metastatic lesion of human prostatic carcinoma. The LNCaP cells grow readily in vitro (up to 8 x 10^5 cells/sq cm; doubling time, 60 hr), form clones in semi-solid media, and show an aneuploid (modal number, 76 to 91) human male karyotype with several marker chromosomes. The malignant properties of LNCaP cells are maintained. Athymic nude mice develop tumors at the injection site (volume doubling time - 86 hr). Functional differentiation is preserved: both cultures and tumors produce prostatic acid phosphatase (PAP) and prostate specific antigen (PSA). High-affinity specific androgen receptor is present in the cytosol and nuclear fractions of cells in culture and in tumors. The model is hormonally responsive: in vitro, 5α-dihydrotestosterone modulates cell growth and stimulates acid phosphatase production. In nude mice, the frequency of tumor development and the mean time of tumor appearance are significantly different for either gender.

LNCaP cells therefore meet criteria of a versatile model for immunological studies of human prostatic cancer in the laboratory. Other prostatic cell lines (3,4) fail to maintain some of the markers characteristic of prostatic epithelium and malignant prostatic cells: e.g., production of secretory human prostatic acid phosphatase (3,5), organ specific prostate antigen (6), responsiveness to androgens (5,7) or the presence of the Y chromosome (7,8). Such cells may not be optimally representative in their antigenic make-up of the majority of prostatic tumors as seen by the clinician and pathologist.

Our aim was to obtain and characterize a stable murine hybridoma cell line secreting mono-

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clonal antibodies reactive with an epitope
present on membrane associated, non-secretory
putative antigen of human prostatic cancer. The
LNCaP cells and partially purified LNCaP plasma
membranes were used as immunogen.

MATERIALS AND METHODS

Hybridomas. 7E11 and 9H10 uncloned hybrid-
oma cultures were produced by S. Leong (Leong,
Kawinski and Horoszewicz - manuscript in prepara-
tion) by fusion of murine myeloma (P3 x 63Ag
8.653) with spleen cells of LNCaP immunized mice
according to standard procedure (9). Both
hybridomas were cloned twice by the limiting
dilutions method (10). After cloning, stable
hybridomas producing MoAb were expanded and
cryopreserved.

Ascites Fluid Production. Hybridoma cells
(4x10⁶) for ascites fluid production were inject-
ed into the peritoneal cavity of female nude
mice. Ascites fluid containing 3 to 8 mg/ml of
MoAb was harvested 4-5 weeks after hybridoma cell
injection.

MoAb Purification and Preparation of Anti-
body Fragments. Whole MoAb molecules were puri-
fied from murine ascites fluid on Affi-gel
protein-A agarose (Bio-Rad) following manufac-
turer's recommendations. To prepare antibody
fragments molecular sieving on Sephacryl 200
(Pharmacia) of affinity purified immunoglobulins
was performed. Appropriate fractions were con-
centrated, digested with pepsin, rechromato-
graphed on Affi-gel protein A-agarose (to remove
the Fc fragments and undigested whole MoAb mole-
cules), separated on Sephacryl 200 and concen-
trated by pressure dialysis. The immunological
activity of ascites fluid vs. purified $F(ab')_2$
fragments was compared in ELISA. Activity of
$F(ab')_2$ was preserved. The overall purity and
molecular seizes of whole antibodies and $F(ab')_2$
was confirmed by polyacrylamide gel electro-
phoresis (PAGE) using 10% gels.

Cell Lines. Thirty-three cell lines of
human origin were used (Table 1). Six cell lines
were isolated and established in our laboratory:
LNCaP (1), TT (11), PAC (12),3G-9, MLD (13) and
SM; 2 cell lines were from American Type Culture
Collection: MDA-MB-23 and FL; 9 cell lines were
obtained from J. Fogh of Memorial Sloan-Kettering
Institute: DU-145, PC-3, MCF-7, MeWo, RT-4,
HT-29, A-209, SAOS-2 and 5959; the remaining 16
cell lines were provided by R. Baker, K. Chadha,
W. Dembinski and M. Ito of RPMI and include:
5637, SK, COLO-205, HeLa-531, HeLa-CCL2, SW-872,
HT-1080, GM-2504, HBC, A-549, CHAGO, SKMES, PC-1,
PC-9, PC-14 and T-24. Murine myeloma line P3 x
63Ag 8.653 was from L. Papsidero of RPMI. All of
the cell lines were routinely maintained in RPMI
medium 1640 supplemented with 10% heat inacti-
vated fetal bovine serum, 1 mM L-glutamine, and
50 µg/ml of penicillin and streptomycin (Gibco).

Human Specimens. Fresh normal and tumor
tissues were obtained either from the Department
of Surgery or the Department of Pathology at
RPMI. The tissues were quick frozen in M-1
embedding matrix (Lipshaw) and stored at -80°.
Human sera were from Blood Bank, from the Depart-
ment of Laboratory Medicine and from the Depart-
ment of Urology at RPMI.

Indirect Immunoperoxidase Staining. Cyto-
spin smears of cultured cells, frozen sections
(4 um thick) and sections of formalin fixed,
paraffin embedded human tissues were used for
immunoperoxidase staining as described previous-
ly (14,15). The intensity of the immunospecific
staining was evaluated using Zeiss microscope
(40 x objective; 10 x ocular).

Isolation of Plasma Membrane-Enriched
Fractions. Plasma membrane-enriched fractions
were obtained from LNCaP cells and normal human
diploid fibroblast (strain MLD) by modification
of published methods (16).

The enzyme-linked immunosorbent assay
(ELISA) has been used for general enzyme immuno-
assay of antigen (17) and screening for MoAb
production (18) using viable and fixed cells, as
well as purified plasma membranes.

RESULTS

Cloning of Hybridomas. Hybridomas 7E11 and
9H10 were cloned twice by the limiting dilution
method (10). Two stable monoclonal (p<0.005)
hybridoma cell lines were obtained and designated
as 7E11-C5 and 9H10-A4 respectively.

Immunospecific Staining. The indirect
immunoperoxidase staining of formalin fixed
LNCaP cells by supernatants from either of the
two cloned hybridoma cultures was positive in
dilutions ranging from 1:200 to 1:300 while
ascitic fluids harvested from mice stained LNCaP
smears at dilutions from 1:50,000 to 1:400,000.
The localization of immunoperoxidase staining of
LNCaP cells differed for MoAb 7E11-C5 and MoAb
9H10-A4. MoAb 7E11-C5 staining was apparent
over the cytoplasmic region with intensity
slightly increasing toward the cell periphery.
MoAb 9H10-A4 produced continuous, narrow band of
strong staining limited only to plasma membrane.
The staining pattern of LNCaP cells from culture,
as well as cells taken directly from nude mouse
tumors was constant for each MoAb.

Viable LNCaP cells when stained by the in-
direct immunofluorescence method showed bright
peripheral rings after exposure to MoAb 9H10-A4.
No staining of viable cells, however, was seen
with MoAb 7E11-C5.

Reactivity of Soluble vs. Sedimentable Cell
Components. Immunoblotting and ELISA using as
antigen the insoluble, membrane rich fraction
from LNCaP cells were strongly positive with both
MoAb 7E11-C5 and 9H10-A4. On the other hand,
neither MoAb reacted in these tests with soluble

cellular LNCaP components such as whole cytosol or purified PAP or PSA when examined according to described methods (19,20).

Other Cell Lines. In addition to LNCaP cultures, 32 human normal and malignant cell lines were evaluated as to their reactivity with both studied MoAbs. None of these cell lines reacted in either ELISA or indirect immunoperoxidase staining regardless of fixation (Table 1).

Isotyping. MoAb 7E11-C5 and 9H10-A4 are of the IgG-1 subclass, as determined by double diffusion gel precipitation with isotype specific antisera (Miles). Consistent with this finding were observations that Protein A conjugated with either fluorescein or horseradish peroxidase (Bio-Rad) failed to react with smears of LNCaP cells following incubation with either MoAb.

Biological Activity. No biological activity of MoAbs 7E11-C5 and 9H10-A4 was detected in vitro nor in vivo: the MoAbs either alone (as 1:5 dilutions of hybridoma supernatants, or 1:100 dilutions of ascites) or in the presence of rabbit complement (1:20) had no measurable growth inhibitory or cytotoxic effects on LNCaP cultures; neither the growth of LNCaP tumors in nude mice (3 groups, 6 animals each) was affected by weekly injections of 1 mg of ascites derived MoAbs 7E11-C5 or 9H10-A4 over a period of 10 weeks, when compared with PBS injected controls.

Distribution in Human Tissues of Antigens Reactive with MoAbs 7E11-C5 and 9H10-A4. A survey of human normal and neoplastic tissues obtained from biopsy, surgery and autopsy was performed to assess the localization of antigens reactive with both MoAbs. Fresh frozen sections fixed in 2% neutral formaldehyde were stained by the indirect immunoperoxidase method and evaluated. Results from observations made on 175 specimens are shown in Table 2.

MoAb 7E11-C5 stained both malignant and apparently normal prostatic epithelial cells with remarkable selectivity. No reactivity was seen in stromal components such as fibers, vessels, muscles, etc. Positive cells stained stronger toward the cell periphery. The staining showed a small degree of heterogeneity among individual cells. A difference was noted in the intensity of staining between normal and neoplastic epithelium. The staining of CaP cells was strong in 9 out of 11 specimens and of moderate intensity in the remaining 2. Apparently normal and hypertrophic prostatic glands showed faint (in 12 out of 16 specimens) to moderate (2 out of 16) staining. Two specimens from benign prostatic hypertrophy (BPH), which were classified as negative, contained only very few rudimentary structures reminiscent of prostatic ducts. Overall, 25 out of 27 specimens from prostates and CaP reacted with MoAb 7E11-C5.

Despite strong staining of cytoplasmic membranes of LNCaP cells, MoAb 9H10-A4 failed to react in frozen sections with either normal pro-

TABLE 1

REACTIVITY OF MoAb 7E11-C5 AND MoAb 9H10-A4 WITH CULTURED HUMAN CELLS BY ELISA AND IMMUNOPEROXIDASE STRAINING

Human Cells in Culture		Reactivity with	
		MoAb 7E11-C5	MoAb 9H10-A4
LNCaP	- Prostatic Ca	+++	++++
DU145	- Prostatic Ca	-	-
PC-3	- Prostatic Ca	-	-
RT-4	- Bladder Ca	-	-
5637	- Bladder Ca	-	-
MCF-7	- Bladder Ca	-	-
MDA-MB-231	- Breast Ca	-	-
HT-29	- Colon Ca	-	-
SK	- Colon Ca	-	-
COLO205	- Colon Ca	-	-
PAC	- Pancreatic Ca	-	-
TT	- Medullary Thyroid Ca	-	-
MeWo	- Melanoma	-	-
SM	- Melanoma	-	-
HeLa-S31	- Uterine Ca	-	-
HeLa-CCL2	- Uterine Ca	-	-
A209	- Rhabdomyosarcoma	-	-
SW872	- Liposarcoma	-	-
HT1080	- Fibrosarcoma	-	-
5959	- Osteogenic Sarcoma	-	-
SAOS-2	- Osteogenic Sarcoma	-	-
H8C	- Bronchogenic Ca	-	-
A549	- Lung Adeno Ca	-	-
CHAGO	- Large Cell Lung Ca	-	-
SKMES	- Squamous Cell Lung Ca	-	-
PC-1	- Lung Ca	-	-
PC-9	- Lung Ca	-	-
PC-14	- Lung Ca	-	-
T-24	- Lung Ca	-	-
MLO	- Normal Fibroblasts	-	-
BG-9	- Normal Fibroblasts	-	-
GM2504	- Normal Fibroblasts	-	-
FL	- Human Amnion	-	-

static epithelium or with neoplastic cells.

Neither MoAb 7E11-C5 nor MoAb 9H10-A4 stained fresh frozen sections from any of the 26 specimens representing 11 different histological types of human non-prostatic tumors.

Among 122 individual specimens from 28 different normal human organs and tissues, 120 did not show any staining with MoAb 7E11-C5. In 2 instances (out of 14) of normal kidneys, poorly defined, low intensity, diffuse and uneven brownish deposits were detected on the inner surfaces and in the lumen of some of the Henle's loops. Pre-incubation of fixed sections with 1% albumin or gelatin solutions reduced such "staining". Similar reactions in the human kidney by the immunoperoxidase staining with various murine monoclonal antibodies were noted by other authors (21,22), but the significance, if any, or the

specificity of such "staining" is at present un-
clear. Again, MoAb 9H10-A4 did not react with
any of the 122 specimens from normal organs.

Development of Competitive Binding ELISA.
After incubation of MoAb 7E11-C5 at appropriate
concentrations (20-100 ng/ml) with whole LNCaP
cells, hypotonic cell lysates, LNCaP cell
sonicates or partially purified plasma membranes,
the original activity of MoAb 7E11-C5 as
measured by ELISA was significantly and
reproducibly reduced. The inhibition was a
function of antigen concentration and the length
of incubation time (results not shown). These
observations suggested that MoAb 7E11-C5 re-
active antigen could also be detected, if present,
in human sera using an appropriately designed assay.

Initial experiments were focused on the
assay specificity and methodology. For these
studies, 3 sera from CaP patients inhibiting
MoAb 7E11-C5 in competitive binding ELISA were
used. Centrifugation (2 hrs; 100,000 x g) failed to
sediment their inhibitory activity which suggested
that the "inhibitor" in serum was not associated
with circulating whole CaP cells, membrane
vesicles or cell fragments, but represented the
MoAb 7E11-C5 reactive epitope in a soluble form.
This observation was unexpected since high speed
centrifugation of either disrupted LNCaP cells,
or spent LNCaP cell culture media yields anti-
MoAb 7E11-C5 directed reactivity only in sedi-
mentable fractions, indicating that the MoAb
7E11 specific epitope was associated with in-
soluble supramolecular aggregates. The level of
competitive binding ELISA inhibitory activity
against MoAb 7E11-C5 in human sera remained
constant after 10 cycles of repeated freezing
and thawing, heating to 56° for 30 min., 6 months
storage at -80°, as well as after overnight in-
cubation at 37° regardless of addition of pro-
tease inhibitors.

ELISA inhibitory activity was not due to the
presence in tested sera of a human antibody with
specificity similar to MoAb 7E11-C5, which could
competitively block available antigenic sites on
the LNCaP detector cells, nor were enzymatic
activities of serum affecting the antigenic sites
of LNCaP cells. This was shown by preincubation
(up to 72 hrs.) of wells containing LNCaP cells
with either "inhibitory" serum, non-inhibitory
serum or PBS. The serum was then removed and
MoAb 7E11-C5 activity was tested by standard
ELISA procedure. No reduction in reaction in-
tensity was observed between control wells and
wells pre-incubated with inhibitory sera.

In addition, either the presence in sera of
anti-murine IgG capable of binding MoAb 7E11-C5
or the existence of an unusual proteolytic activ-
ity directed against monoclonal antibodies in
general, was excluded by preincubation of in-
hibitory sera with murine MoAb 9H10-A4 and show-
ing that immunologic reactivity with LNCaP cells
and membranes was unaffected.

TABLE 2
ANTIGEN IN FROZEN SECTIONS FROM 175
SPECIMENS DETECTED BY INDIRECT IMMUNOPEROXIDASE
STAINING WITH MoAbs 7E11-C5 AND 9H10-A4

	Positive/Total Tested	
	MoAb 7E11-C5	MoAb 9H10-A4
Human Prostatic Epithelium	Reactive	Reactive
CaP foci in prostate	9/9	0/9
CaP metastases in lymph nodes	2/2	0/2
Benign prostatic hypertrophy	5/7	0/7
Normal prostates	9/9	0/9
Human Tumors (Non-Prostatic)		
Breast Ca	0/8	0/6
Renal Cell Ca	0/3	0/3
Bladder Ca	0/2	0/2
Adrenal Ca	0/2	0/2
Colon Ca	0/2	0/2
Sarcoma	0/2	0/2
Squamous Cell Ca	0/3	0/3
Melanoma	0/1	0/1
Neuroblastoma	0/1	0/1
Uterine Ca	0/1	0/1
Pancreatic Ca	0/1	0/1
Normal Human Organs		
Urinary Bladder	0/5	0/5
Urether	0/5	0/5
Seminal Vesicles	0/3	0/3
Testis	0/4	0/4
Kidney	2/14	0/14
Ovary	0/3	0/3
Uterus	0/3	0/3
Breast	0/3	0/3
Bronchus	0/4	0/4
Lung	0/5	0/5
Liver	0/7	0/7
Spleen	0/8	0/8
Pancreas	0/5	0/5
Tongue	0/2	0/2
Esophagus	0/1	0/1
Stomach	0/3	0/3
Small Intestine	0/3	0/3
Colon	0/9	0/9
Thyroid	0/5	0/5
Parathyroid	0/1	0/1
Adrenals	0/4	0/4
Lymph Node	0/5	0/5
Skeletal Muscle	0/5	0/5
Heart	0/5	0/5
Aorta	0/3	0/3
Vena Cava	0/3	0/3
Brain	0/1	0/1
Skin	0/4	0/4

Next, the possibility was investigated that
"inhibitors" in positive CaP sera were unspecific
and interacted only with the Fc portion of MoAb
7E11. To this end, the inhibition of immuno-
reactivity of 7E11 F(ab')₂ antibody fragments by
CaP sera was tested. The F(ab')₂ antibody frag-
ments were as susceptible to inhibition by posi-

TABLE 3

SUMMARY TABLE OF MoAb 7E11-C5 COMPETITIVE BINDING ELISA IN HUMAN SERA

Serum Source	Number Tested	SR 7E11	Positive	
Prostatic Cancer (CaP)	43		20	(46.5%)
Benign Prostatic Hypertrophy (BPH)	7		0	
Non-Prostatic Malignancies	66		3	(4.6%)
Normal Blood Donors	30		0	
Total	146		23	

Two tail Fisher Exact Probability Test indicates that there is a significantly higher SR_{7E11} positive rate (p <0.0001) in a population of 43 CaP patients as opposed to a group of 103 non-CaP-controls (normal, BPH and other malignancies). The assays were blinded.

tive human sera from CaP as were the complete MoAb 7E11-C5.

Taken together, the above experiments indicate that observed ELISA inhibition results from specific immunological reaction between MoAb 7E11 and corresponding antigen present in serum from some CaP patients.

The assay methodology for testing human sera from normal blood donors, non-prostatic malignancies and patients with prostatic cancer for specific binding of MoAb 7E11-C5 in limiting concentrations was established as follows:

Aliquots (125 µl) of serum were incubated (3 hrs., room temp.) with:

a) 125 ul of diluent (PBS with 0.3% bovine serum albumin, pH 7.2, sodium azide 0.05%)

b) 125 ul of MoAb 7E11 (60 ng/ml in diluent)

c) 125 ul of MoAb 9H10 (6 ng/ml in diluent)

As references of total MoAb activity in the absence of serum, MoAb 7E11-C5 (30 ng/ml) and MoAb 9H10-A4 (3 ng/ml) in diluent only were used. In addition, each microtiter plate contained a set (12 wells) of external controls consisting of normal female serum preincubated separately with each MoAb and diluent.

The reaction mixtures were then incubated in a single 96 well microtiter plate (Falcon) overnight (18 hrs, 4°C; quadruplicate wells, 50 ul/well) with air dried LNCaP cells ($4x10^4$ cells/well, 2.0% formaldehyde fixed for 30 min) to determine reactivity by ELISA. The results of the ELISA test (O.D. read at 490 nm) are expressed as the Specific Reactivity with MoAb 7E11-C5 factor (SR_{7E11} factor). The SR_{7E11} factor is calculated according to formula:

$$SR_{7E11} = \frac{O.D. \ (7E11+diluent)}{O.D. \ (7E11+serum)} \times \frac{O.D. \ (9H10+serum)}{O.D. \ (9H10+diluent)}$$

The inclusion of MoAb 9H10 in the test allows to compensate for potential differences in kinetics of binding of MoAb to target LNCaP cells in high (50%) serum concentration, as well as for unexpected presence in individual sera of interfering macromolecules (anti-murine IgG, enzymes, etc.). The MoAb 9H10-A4 strongly binds to LNCaP plasma membranes, but is unrelated in specificity to MoAb 7E11-C5 and does not react with other human cell lines, or frozen sections of normal human organs or malignant tumors. Neither normal nor CaP sera inhibit specifically MoAb 9H10-A4.

Survey of Human Sera by Competitive Binding ELISA. To establish the average numerical value of SR_{7E11} factor for normal, healthy individuals, 30 sera from RPMI Blood Bank donors were tested. The mean SR_{7E11} of this group was 1.13 ± 0.23 ($\bar{x}$ ± S.D.). No significant differences between the mean values of the SR_{7E11} factor for groups of males and females were found. For the threshold defining positive results (at the p<0.01 level), $\bar{x}$ + 3 S.D. was calculated to be 1.82. The value above 1.82 for SR_{7E11} was used for the classification of Specific Reactivity as positive.

Subsequently, additional 116 sera were tested: 43 from CaP patients, 7 from individuals with benign prostatic hypertrophy and 66 sera from nonprostatic malignancies. Tables 3, 4, and 5 show the results. A strong statistical correlation emerged between the assay positive outcome and diagnosis of prostatic cancer. In addition, the patients with positive SR_{7E11} were more likely to be in progression than those who were negative. Similarly, a higher percentage of positive tests were among patients with widely disseminated disease vs. less advanced clinical stages. Among 66 sera from individuals with tumors of nonprostatic origin, only 3 (4.6%)

TABLE 4

MoAb 7E11-C5 COMPETITIVE BINDING FI A IN PROSTATIC CANCER

Clinical Evaluation	Number Tested	SR 7E11	Positive
No Apparent Disease	7	0	
Remission/Stable	13	6	(46%)
Progression	23	14	(61%)
Total	43	20	

CaP Stage			
B I	2	0	
B II	5	1	(29%)
C I	3	2	
D I	4	1	
D II	29	16	(55%)
Total	43	20	

Logistic regression relating the probability that the patient was
in CaP progression to the SR$_{7E11}$ indicates a significant (at p<0.05)
relationship. Patients with positive SR$_{7E11}$ are more likely to be
in progression, than those who are negative. The assays were blinded.

tested positive (Table 5). Two of the positive
sera were from females with disseminated uterine
and renal carcinomas respectively. The third
positive serum was obtained from young male with
testicular embryonal carcinoma.

DISCUSSION

Monoclonal antibodies (MoAb) obtained by the
hybridoma technology are potentially powerful
tools for cancer detection, diagnosis and therapy
So far, the success in developing reagents that
are exclusively tumor specific has been limited.
Possibly, the low frequency, poor accessibility
or, perhaps, even complete absence of tumor
specific epitopes is responsible. The development
of diagnostic and therapeutic reagents against
neoplasms derived from cells expressing organ
(or tissue) specific antigens, appears to offer
an immediate and practical alternative.

Prostatic epithelium has limited distribu-
tion, may not carry out functions vital for the
survival of a cancer patient, but was already
shown to produce organ specific, albeit secretory
macromolecules. Prostatic organ specific mole-
cules preserved on neoplastic cells and bound to
membranes could be targeted by MoAb as a thera-
peutic approach. Cancer of the prostate is the
second most frequent tumor of males in the
United States (23), claiming annually over 25,000
lives. Unknown etiology, variable pathology,
intricate relationship to endocrine factors and
anaplastic progression contribute to the com-
plexity of this disease and limited effective-
ness of available therapies.

TABLE 5

MoAb 7E11-C5 COMPETITIVE BINDING ELISA IN
HUMAN SERA FROM NON-PROSTATIC MALIGNANCIES

Diagnosis	SR$_{7E11}$ Positive / Total Tested
Testicular Tumors Embryonal Ca	1/16
Transitional Cell Ca (Bladder)	0/7
Renal Cell Ca	1/4*
Breast Ca	0/3
Ovarian Adeno Ca	0/3
Uterine Adeno Ca	1/2*
Gastric Ca	0/3
Hepatoma	0/2
Pancreatic Adeno Ca	0/3
Colon and Rectum Adeno Ca	0/3
Lung Ca	0/3
Sarcoma	0/4
Astrocytoma, Chordoma	0/2
Squamous Cell Ca	0/3
Basal Cell Ca	0/2
Histiocytoma	0/1
Mesothelioma	0/1
Lymphoma, Leukemia	0/4
Total	3/66 (4.6%)

*SR$_{7E11}$ positive sera were from terminal patients who
expired shortly after testing.

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The progress toward establishing effective
immunological methods for detection and success-
ful management of CaP may depend on laboratory
experimentation with most suitable models used
as reagents for MoAb production. Prostate cancer
specific antigen may not have been yet defined
by monoclonal antibodies, although several CaP-
associated epitopes were already described
(24-33).

Several MoAb are available against two well-
characterized, purified to homogeneity, soluble
glycoproteins produced and secreted by either
normal or malignant human prostatic epithelium.
PSA (24) is present in human prostate epithelium,
seminal plasma and CaP cells. Readily produced
polyclonal and monoclonal antibodies to purified
PSA (6,19) established this antigen as a sero-
diagnostic marker for CaP, marker for human
prostatic epithelial cells and immunohistologic
marker for prostate neoplasms. Another
organ specific, well known marker protein of
normal and neoplastic human prostatic cell is
human prostatic acid phosphatase. PAP (25) is a
glycoprotein with m.wt. 100,000 and established
aminoterminal sequence and carbohydrate composi-
tion (26). Murine monoclonal antibodies (20,27)
identify 3 distinct antigenic determinants and
several sensitive immunoassays to measure PAP
were developed. Experiments by Lee et al. (28)
with LNCaP model system suggest that monoclonal
anti-PAP antibody has potential for antibody-
directed radio-imaging and MoAb targeted chemo-
therapy of prostate cancer. Both PSA and PAP
are secretory products of diagnostic value and
could be detected not only in cells but also in
plasma of patients with advanced CaP, nude mice
bearing LNCaP tumors and in LNCaP culture super-
natants. PSA and PAP solubility and secretion
could impair the intracellular retention of
directed at them antibodies and diminish the full
pharmacologic effectiveness of cytotoxic con-
jugates.

Another strategy of MoAb production against
human prostatic cancer cells has been the utili-
zation as immunogens of whole cells or fraction-
ated cell preparations from established in vitro
cultures of human malignant prostatic cells PC-3
and DU145. A variety of generated MoAb have
shown reactivity not only with cell surface or
cytoplasmic antigens of CaP cells, but also with
cells from other malignancies and most important-
ly, with several non-prostatic normal human
tissues (21,22,29-33).

In this report, we describe the isolation
of two stable murine hybridomas secreting MoAb
directed against LNCaP cells which were used as an
immunogen. The LNCaP cells originated from a
metastasis of prostatic cancer and maintain in
vitro biologic properties as well as several bio-
chemical markers characteristic of human malignant
prostatic epithelium (1,2). Studied by us, MoAb
7E11-C5 and MoAb 9H10-A4 were of the IgG1 sub-

class and as such, either alone or with comple-
ment, lacked detectable biological activities
against LNCaP cells in vitro or in nude mice.
Both MoAbs reacted in ELISA and by immunoblotting
with sedimentable, cytoplasmic membrane rich
fractions of LNCaP cells, but not with soluble
cytosol or secretory glycoproteins such as PSA
or PAP.

MoAb 9H10-A4 had specificity restricted to
epitopes present on the surface of LNCaP cell
plasma membrane as demonstrated by ELISA and
immunospecific staining of a variety of viable
or fixed cells and frozen sections. No binding
of MoAb 9H10-A4 was detected to any other than
LNCaP human prostatic and non-prostatic normal
or malignant cells in studies involving 32 cell
lines, 27 prostates and 148 other fresh-frozen
specimens of human organs, normal tissues and
tumors. This suggests that MoAb 9H10-A4 defined
antigen could be unique for an individual pros-
tatic tumor or perhaps even a single metastasis
from which the LNCaP cells were isolated.
At present, MoAb 9H10-A4 remains as a use-
ful reagent to positively identify LNCaP cells
and distinguish them from other cultured cells.
In addition, this MoAb serves as a reliable
control in competitive binding ELISA with MoAb
7E11-C5 for detection of circulating antigens
associated with CaP.

MoAb 7E11-C5 reacted with epithelial cells
in frozen sections from prostatic carcinoma,
benign prostatic hypertrophy and to a lesser
degree with normal prostatic glands. Among 33
grown in vitro normal and neoplastic cell lines,
only LNCaP cells bound MoAb 7E11-C5 in ELISA and
in indirect immunospecific staining of dried and
fixed smears. It is of interest that CaP de-
rived DU-145 and PC-3 cells did not exhibit any
reactivity with MoAb 7E11-C5. This finding
parallels the absence or diminution of pheno-
typic expression in PC-3 and DU-145 of other
marker molecules (PAP, PSA, androgen receptors)
which are characteristic of human epithelial pro-
static cells and are abundantly preserved in
LNCaP cultures (3,5,6,7). Strong reactivity of
MoAb 7E11-C5 with LNCaP membrane preparations and
fixed cells contrasted sharply with the lack of
staining by the indirect immunofluorescence
method of viable, unfixed LNCaP cell suspensions.
This observation suggests that epitopes specific
for MoAb 7E11-C5 are either absent or not avail-
able for binding on the outer surface of living
LNCaP cells. It remains to be determined whether
such restriction applies to normal and malignant
viable cells from human prostates. The results
of such experiments could help to project the
practical potential of appropriate MoAb 7E11-C5
conjugates as either imaging or therapeutic
agents for CaP.

The evidence for selective specificity of
MoAb 7E11-C5 for human prostatic epithelium was
reinforced by consistently negative results of

immunospecific staining of numerous fresh frozen
sections from a wide range of human nonprostatic
normal or malignant tissues. Noted on a couple
of occasions, poorly defined staining of kidney
tubules require additional observations to ascer-
tain its reproducibility and specificity on a
larger size sample of fresh biopsy specimens.

At present, we have no information on the
molecular nature of epitopes reactive with MoAb
7E11-C5. In cultured LNCaP cells, these epitopes
are strictly associated with non-soluble, sedi-
mentable material. In contrast, the serum of many
CaP patients contains such epitopes in a soluble
form. Perhaps pathways of processing macro-
molecules in vitro vs. in vivo during synthetic
or autolytic events are responsible for this
dichotomy. The results of a competitive binding
ELISA establishing a statistical link between CaP
and positive tests for circulating epitopes are
encouraging. The sensitivity and specificity of the
described assay is likely to be improved, when
instead of a dried cell suspension a defined amount
of purified and standardized antigen is used. In
addition, when such antigen is available, the
issue of precise quantitation of MoAb 7E11-C5
reactive molecules in human sera could be mean-
ingfully addressed and correlations with CaP
stages better delineated. We felt that reporting
in this paper an early and developmental stage of
a new test, attempts to quantitatively describe
each positively testing individual in terms of
arbitrary units were premature.

Additional experiments should define the
future of MoAb 7E11-C5 and 9H10-A4 in diagnosis
and management of human prostatic cancer.

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Western Blot Assay for Prostate-Specific Membrane Antigen in Serum of Prostate Cancer Patients

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ABSTRACT: There is a need for the development of new diagnostic tools for the early detection of prostate cancer. A candidate molecule for a new screening test is a prostate-specific membrane antigen (PSM) recognized by the monoclonal antibody 7E11.C5. We carried out studies aimed at identifying PSM in the serum of normal and benign prostatic hyperplasia (BPH) donors and patients with adenocarcinoma of the prostate, in order to judge whether the development of a serum assay using this marker was feasible. By Western blotting, we found significant levels of PSM in serum samples from prostatic cancer patients, in the seminal fluid of pooled normal donors, in BPH patients, and in normal male sera. Similar to prostate-specific antigen (PSA), PSM was present in seminal plasma in higher concentrations than in serum, and PSM levels in prostatic cancer patients were significantly higher than in normal controls. These data suggest that the development of an assay utilizing the PSM and new monoclonal antibodies directed against the antigen, could provide a feasible test for prostatic cancers.   © 1994 Wiley-Liss, Inc.

INTRODUCTION

Prostate cancer has become a major concern within the aging male population in the United States. With the advent of strenuous diagnostic strategies and periodic testing of nonsymptomatic males over the age of 50, a greater number of early stage cases of adenocarcinoma of the prostate are being diagnosed [1]. However, it has been argued that the diagnostic protocols to which patients are being subjected are too invasive for the number of new cases detected [1].

The development of a new prostate cancer screening test which could potentially be used along with prostate-specific antigen (PSA) levels would afford an increase in screening confidence and a reduction in false positive results [1]. A candidate molecule for the development of a new screening assay is a prostate membrane-associated recognized by the 7E11.C5 antibody [2]. This antibody has been successfully used for in vivo imaging of metastatic prostatic carcinoma [3,4], and the nucleotide sequence of a cDNA coding

for the antigen has been determined [5]. The antigen is a transmembrane protein based on the deduced amino acid sequence, and has been termed prostate-specific membrane antigen or PSM [5]. The 7E11.C5 antibody reacts with antigen localized in subcellular organelles as well as in the plasma membrane [6]. We report here direct evidence for the increased levels of PSM in sera from patients with adenocarcinoma of the prostate, and also its presence in pooled seminal plasma from normal males.

MATERIALS AND METHODS

Cell Lines

The cell lines DU-145 and PC-3 were obtained from the American Type Culture Collection. The LNCaP

cell line established by J.S. Horoszewicz et al. was utilized [7,8]. All three cell lines were grown in RPMI 1640 medium supplemented with L-glutamine, 10% fetal calf serum (Gibco-BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a 5% CO₂/95% air incubator. In addition, monoclonal antibodies 7E11.C5 (CYT-351) and 9H10 [2] were purified from mouse ascites, on a protein A Sephadex column were obtained from Cytogen Corporation, Princeton, NJ.

LNCaP-Based ELISA Assay

We tested for the presence of the PSM antigen in the sera of patients with adenocarcinoma of the prostate using a modification of a previously described LNCaP-based ELISA assay [2]. Briefly, LNCaP cells grown to confluence, harvested in PBS, and resuspended at 1.5 × 10⁷ cells/ml in 3 mM HEPES (pH 7.4), 0.3 mM MgCl₂, 0.5 mM CaCl₂, and 0.5 mM AEBSF (4-(2-Aminoethyl)-benzensultonylfluoide, HCl) (CalBiochem, La Jolla, CA). Twenty microliters of cell suspension (3 × 10⁵ cells) were added to the center of a well (96-well flat-bottomed Immulon-4 plate, Dynatech Laboratories, Chantilly, VA) and dried at 37°C. Once dry, the cells were fixed for 30 min at room temperature with 2% formaldehyde (pH 7.4), and washed three times with PBS, and the plates were blocked for 2 hr at room temperature with 1% Type A (300 Bloom) porcine skin gelatin (Sigma Chemicals, St. Louis, MO) in PBS (with 0.5% sodium azide). When necessary, the plates were stored at 4°C, and warmed to 37°C prior to use. 250 μl aliquots of normal or patient sera were individually mixed with 7E11.C5 (13 ng/ml final), 9H10 (15 ng/ml final), or PBS for 3 hr at room temperature. 50 μl of the antibody/serum sample were added to wells containing the fixed cells, and incubated overnight at 4°C. Plates were washed extensively, and HRP-labeled goat anti-mouse secondary antibody (Sigma) was added for 1 hr at room temperature, followed by extensive washing and addition of 200 μl of color developing solution (50 mM sodium acetate, 2 mM OPD (o-Phenylenediamine), 0.08% H₂O₂, pH 6.0), and the reaction was stopped after 35 min with 50 μl 2.5 N sulfuric acid.

This competitive-inhibition ELISA assay measures the relative loss of activity when antibody is mixed with human serum vs. antibody mixed with diluent (in this case PBS). We express relative inhibition (RI) as the activity of the antibody in the presence of diluent divided by the activity of the antibody in the presence of serum. The greater the concentration of antigen in the serum sample, the greater the RI value will be, as the activity in the presence of serum will be

lower than that found with the diluent. As control for nonspecific binding of the monoclonal antibody by serum proteins, the 9H10 monoclonal antibody is used. This antibody recognizes a protein present on the surface of LNCaP cells and has not been detected in the frozen sections of any human tissues studied thus far [2]. The 7E11.C5 and 9H10 monoclonal antibodies were generated from the same mouse, and are of identical IgG subclass. Specific reactivity (SR) is defined as the RI value found using the 7E11.C5 antibody divided by the RI value found using the 9H10 antibody.

Cell Lysates

Cultured cells were grown to confluency, rinsed twice with warm PBS, once with 0.05% trypsin, 0.02% EDTA, and harvested in PBS after 5 min. Cells (4 × 10⁷/ml) were lysed in 50 mM Hepes (pH 7.5), 10% glycerol, 1% Triton X-100, 15 mM MgCl₂, 1 mM AEBSF, and 1 mM EDTA for 20 min at 4°C. The lysate was centrifuged at a speed of 13,000g for 30 min at 4°C, and frozen at −20°C until further use.

Electrophoresis and Western Blotting

Protein samples (93 μg/lane) were run under reducing conditions on 7% or 8.5% SDS-PAGE, and the separated proteins were blotted on PVDF membrane as described previously [9]. Membranes were then probed with 3 μg/ml 7E11.C5 antibody, 167 ng/ml sheep anti-mouse HRP labeled secondary antibody, and the membrane developed in chemiluminescent developing medium (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Blots were visualized by exposing X-ray film, the image scanned with an HP IIcx Scanjet, and band intensities semiquantitated using Sigma Scan Software. Protein concentration of serum samples was determined using the BioRad protein assay kit.

Samples were assessed as to the strength of the Western blot signal, based on densitometric evaluation of scanned blots by SigmaScan/Image measurement software. Values were equal to ten times the inverse of the resulting peak intensity. All cancer patient samples were assessed against healthy normal donor samples from the same Western blot in a single blind fashion. A minus (−) was assigned to those values below .08. A single plus (+) was assigned to values between 0.081–.240) (mean plus or minus three standard deviations), and was derived from cancer-free patients. A double plus (+ +) was the range from .241–.500, and samples greater than .500 were assigned a triple plus (+ + +).

Clinical Material

The general health of control volunteers (ages 25–35) was confirmed by health history. Stages of cancer C-D_2 were determined by relevant clinical and pathological information, and all had been or were under treatment, e.g., radiation, hormones. Earlier stage patients B_1, B_2 were evaluated prior to treatment, and serum was obtained at that time. Knowledge of the clinical data was unknown to those performing the laboratory tests, until after the data were finalized. For the purposes of this report, further data were not collected, i.e., clinical progression, stable, etc., but will be available in future analyses when significant numbers of patients are accumulated. Benign prostate hyperplasia (BPH) diagnosis was based on clinical history. No BPH patient had received treatment. The degree and/or severity of the clinical condition was not quantitated. Knowledge of the clinical condition was unknown to those performing the assays until after the data was recorded.

RESULTS

Identification of Positive Sera Using the LNCaP-Based Competitive Inhibition ELISA Assay

Sera were initially identified from donors with adenocarcinoma of the prostate using the modified LNCaP-based competitive inhibition ELISA assay [2]. There were 12 sera from donors with stage B_1 to D_2 adenocarcinomas of the prostate, which were positive using the ELISA assay. The specific reactivity (SR) for normal donors was 0.886 ± 0.154 (mean ± SD, n = 45), and the cutoff value for positive results (mean ± 3 SD) was calculated as 1.347. The range of positive values was found to be between 1.40–3.77, and 30% (6/20) of patients with localized prostatic carcinoma (stages B_1 to D_1) and 38.5% (5/13) of donors with metastatic disease (stage D_2) were positive. All control sera (n = 45) were negative in the competitive ELISA assay.

Identification of PSM by Western Blotting

Lysates from cell cultures of LNCaP, DU-145, and PC-3 were electrophoretically separated under reducing conditions and blotted on PVDF membrane. Using 7E11.C5 as the reporting antibody, an immunoreactivity of a band at MS ≈ 110 kDa in lysates of LNCaP was present, but there was no change of significance in lysates of DU-145 or PC-3 (Fig. 1, lanes 1–3). A strong immunoreactive band at ≈ 110 kDa from normal human pooled seminal plasma was found (Fig. 1, lane 4), confirming the presence of PSM in human seminal plasma.
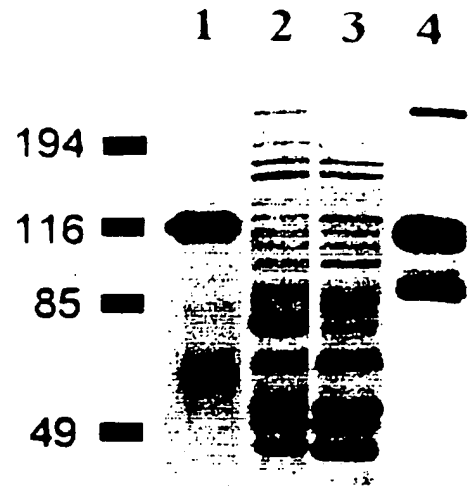
Strong immunoreactivity (+ +) in Western blots

Fig. 1. Western blots of pooled normal seminal plasma and detergent lysates of cultured LNCaP, DU-145, and PC-3 cell lines. Samples were run under reducing conditions on 8.5% SDS-PAGE, followed by electrophoretic blotting on PVDF. The reporting antibody was 7E11.C5 (3 μg/ml). Lane 1, LNCaP lysate; lane 2, PC-3 lysate; lane 3, DU-145 lysate; lane 4, whole pooled human seminal plasma. Molecular markers are expressed in kilodaltons, and PSM is found at ≈ 110 kDa.

was found at the expected molecular weight in 4 of 9 serum samples from donors with localized adenocarcinoma of the prostate (C-D_1), and a faint band was found in 4 of 9 samples (Fig. 2 and Table I). For donors with metastatic disease (D_2), 6 of 7 samples showed strong (+ + to + + +) immunoreactivity, and 1 of 7 showed no immunoreactivity (Fig. 2 and Table I). Several normal male sera also showed a light (+) band at ≈ 110 kDa. Three BPH sera were also reactive (+) to (+ +). Densitometric quantitation of the ≈ 110 kDa immunoreactive band from sera of D_1 and D_2 cancer patients was found to be 3–5 times greater than in sera from normal male donors. Whole sera diluted in loading buffer were not testable under our electrophoretic and blotting conditions, perhaps due to an excessive amount of protein on the gel.

The best delineation of positive bands and an acceptable chemiluminescence signal, along with a reduction in background immunoreactivity, was achieved when serum samples were diluted in lysis buffer. Under these conditions, the serum could be diluted at 1:7 without affecting immunoreactivity.

DISCUSSION

Monoclonal antibody 7E11.C5 recognizes a prostate-specific marker, PSM, of molecular weight ≈ 110 kDa, and may have a potential for being a useful tool in the diagnosis of prostate cancer [2]. In the present
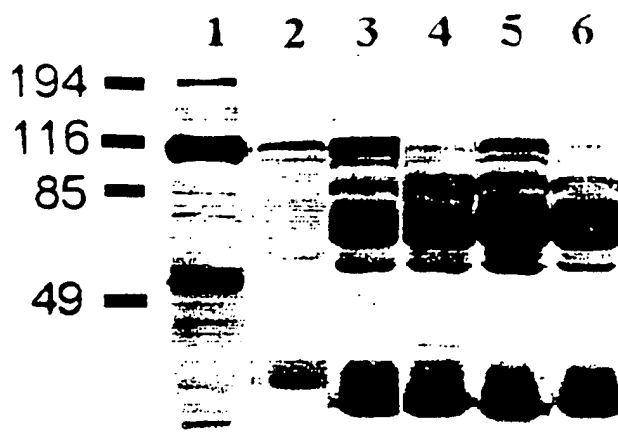
Fig. 2. Western blots of normal and prostatic carcinoma serum run on SDS-PAGE under reducing conditions and electrophoretically blotted on PVDF. The reporting antibody is 7E11.C5 (3 μg/ml). All sera were diluted 1:7 in lysis buffer (see Materials and Methods). Lane 1, undiluted LNCaP lysate; lanes 2 and 3, stage D2 donors; lane 4, sample from a donor with benign prostatic hyperplasia; lane 5, stage D1 donor with a PSA level of 12.2; lane 6, stage D1 donor with a PSA level of 0.2.

TABLE I. Qualitative Analysis of PSM Antigen Levels in Western Blots of Human Diluted Serum*

CaP stage	Number tested	−	+	+ +	+ + +
Normals	7	0	7	0	0
BPH	3	0	2	1	0
C	2	0	2	0	0
D_1	7	1	2	4	0
D_2	7	1	0	2	4
Total	26	2	13	7	4

*Samples were qualitatively judged as to the strength of the Western blot signal, single-blinded. Patient samples are categorized as normals (noncancer donors, under the age of 35), BPH (benign prostatic hyperplasia), or as localized disease (stage C and D_1), or metastatic disease (D_2). The quantitation methods of the Western blots can be found in Materials and Methods.

communication we report an increased serum content of PSM by Western blotting in patients with stages C, D_1, and D_2 prostate cancer. The antibody, modified by the addition of ^{111}In, has been used successfully as an immunoscintigraphic agent in clinical trials in patients with prostatic cancers [3,4]. Attempts at developing a serum assay to detect the presence of PSM have been confounded by the question of whether the antigen is actually present in measurable amounts. Thus, detection of increased PSM in serum of prostate cancer patients by Western blotting supports the feasibility of a serum assay.

In the report describing the LNCaP-based competitive inhibition ELISA assay, a significant increase in 7E11.C5 antigen in 46.5% (20/43) prostatic cancer sera tested was reported [2]. In contrast, only 2.9% (3/103) of noncarcinoma of the prostate samples were positive [2]. Thus, a significantly higher positive rate ($P < 0.0001$, two-tail Fisher Exact Probability Test) in samples from prostatic cancer donors than in normals or other malignancies was noted [2]. Fifty-five percent of donors with metastatic prostatic carcinoma were positive, compared to 29% with localized disease [2]. With the current modification of the LNCaP-based competitive inhibition ELISA assay, similar results were seen, i.e., 38.5% of donors with metastatic prostatic carcinoma and 30% of those with localized disease were positive.

We found strong immunoreactivity by Western blotting of undiluted seminal plasma, confirming earlier preliminary results [10]. Normal male serum and serum from untreated BPH patients contained PSM detectable by Western blotting. The LNCaP-based competitive inhibition ELISA assay, while demonstrating that PSM is potentially of use for the development of a serodiagnostic assay for prostate cancer, is not presently a commercially viable assay. However, it appears that the increase in levels of PSM antigen in serum from prostatic carcinoma patients, compared to normals, is sufficient for the development of a new antibody-based detection assay measuring serum PSM levels. Such an effort, initially focusing on developing a panel of additional second generation monoclonal antibodies suitable for new sandwich-type ELISA assays, is underway.

CONCLUSIONS

The prostate-specific marker PSM, as recognized by the monoclonal antibody 7E11.C5, is present in the serum of patients with adenocarcinoma of the prostate. The levels of this material in serum of prostate cancer patients are significantly higher than in those of normal male donors, as measured by Western blotting techniques, and this warrants the development of a new assay for PSM for detection and diagnosis of prostate cancer.

ACKNOWLEDGMENTS

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Five Different Anti-Prostate-specific Membrane Antigen (PSMA) Antibodies Confirm PSMA Expression in Tumor-associated Neovasculature[1]

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein that was initially characterized by the monoclonal antibody (mAb) 7E11. PSMA is highly expressed in prostate secretory-acinar epithelium and prostate cancer as well as in several extraprostatic tissues. Recent evidence suggests that PSMA is also expressed in tumor-associated neovasculature. We examined the immunohistochemical characteristics of 7E11 and those of four recently developed anti-PSMA mAbs (J591, J415, and Hybritech PEQ226.5 and PM2J004.5), each of which binds a distinct epitope of PSMA. Using the streptavidin-biotin method, we evaluated these mAbs in viable prostate cancer cell lines and various fresh-frozen benign and malignant tissue specimens. In the latter, we compared the localization of the anti-PSMA mAbs to that of the anti-endothelial cell mAb CD34. With rare exceptions, all five anti-PSMA mAbs reacted strongly with the neovasculature of a wide spectrum of malignant neoplasms: conventional (clear cell) renal carcinoma (11 of 11 cases), transitional cell carcinoma of the urinary bladder (6 of 6 cases), testicular embryonal carcinoma (1 of 1 case), colonic adenocarcinoma (5 of 5 cases), neuroendocrine carcinoma (5 of 5 cases), glioblastoma multiforme (1 of 1 cases), malignant melanoma (5 of 5 cases), pancreatic duct carcinoma (4 of 4 cases), non-small cell lung carcinoma (5 of 5 cases), soft tissue sarcoma (5 of 6 cases), breast carcinoma (5 of 6 cases), and prostatic adenocarcinoma (2 of 12 cases). Localization of the anti-PSMA mAbs to tumor-associated neovasculature was confirmed by CD34 immunohistochemistry in sequential tissue sections. Normal vascular endothelium in non-cancer-bearing tissue was consistently PSMA negative. The anti-PSMA mAbs reacted with the neoplastic cells of prostatic adenocarcinoma (12 of 12 cases) but not with the neoplastic cells of any other tumor type, including those of benign and malignant vascular tumors (0 of 3 hemangiomas, 0 of 1 hemangioendothelioma, and 0 of 1 angiosarcoma). The mAbs to the extracellular PSMA domain (J591, J415, and Hybritech PEQ226.5) bound viable prostate cancer cells (LNCaP and PC3-PIP), whereas the mAbs to the intracellular domain (7E11 and Hybritech PM2J004.5) did not. All five anti-PSMA mAbs reacted with fresh-frozen benign prostate secretory-acinar epithelium (28 of 28 cases), duodenal columnar (brush border) epithelium (11 of 11 cases), proximal renal tubular epithelium (5 of 5 cases), colonic ganglion cells (1 of 12 cases), and benign breast epithelium (8 of 8 cases). A subset of skeletal muscle cells was positive with 7E11 (7 of 7 cases) and negative with the other four anti-PSMA mAbs. PSMA was consistently expressed in the neovasculature of a wide variety of malignant neoplasms and may be an effective target for mAb-based antineovasculature therapy.

INTRODUCTION

PSMA[3] is a type II membrane glycoprotein of M_r ~100,000 that was initially characterized by the mAb 7E11 (1, 2). Recent studies have confirmed the location of the PSMA gene on chromosome 11p and have demonstrated the existence of a related PSMA-like gene on 11q (3-5). Two variant forms of PSMA. initially predicted to exist as PSMA. and a spliced form, PSM', have been subsequently confirmed. PSMA is highly expressed in benign prostate secretory-acinar epithelium. prostatic intraepithelial neoplasia, and prostatic adenocarcinoma (2, 6-8), and evidence suggests that PSMA expression is greatest in high-grade and hormone-insensitive cancers (2. 9-11). A shorter. alternatively spliced and presumably cytosolic form of PSMA. named PSM'. is the predominant form expressed in benign prostate epithelium (12, 13). Several studies have shown that anti-PSMA mAbs bind to several nonprostate tissues, including duodenum and kidney (6, 14, 15), and to the vasculature associated with solid malignant tumors (15, 16).

The function of PSMA is currently under investigation. Pinto et al. (17) demonstrated that PSMA has a folate hydrolase-type of activity because LNCaP cells were shown to hydrolyze γ-glutamyl linkages in methotrexate triglutamate. Others have demonstrated that PSMA has a neuropeptidase-type function (18, 19). On the basis of these enzymatic characteristics, the nomenclature committee of the International Union of Biochemistry and Molecular Biology has recommended for PSMA the formal name of glutamate carboxypeptidase (EC 3.4.17.21; Ref. 20).

The 7E11 antibody is a specific murine IgG mAb that was derived after immunization of mice with preparations from the LNCaP human prostate cancer cell line (1). 7E11 has been well characterized and is known to bind an intracellular epitope of PSMA not present on PSM'. As a result, 7E11 does not bind viable prostate cancer cells (1, 16, 21). Modified by the addition of ¹¹¹In, 7E11 is used currently at some centers as an imaging agent in vivo. Clinical trials have demonstrated that this radioimmunoconjugate of 7E11, known as ¹¹¹In-capromab pendetide. may be a useful adjunct in identifying and localizing metastatic or recurrent prostate cancer (22-25).

A number of other anti-PSMA mAbs have been developed recently that bind epitopes that are distinct from that recognized by 7E11 (13, 16). For example, the mAbs J591, J415, J533, and E99 bind to the extracellular PSMA domain (16). Investigators at Hybritech Inc. (San Diego. CA) have identified and purified the mAb PEQ226.5, which binds the peptide backbone of the PSMA extracellular domain. In addition, investigators at Hybritech Inc. have identified PM2J004.5, which binds an epitope of the intracellular PSMA domain that is distinct from that bound by 7E11 (13).

The purpose of this study was to compare the immunohistochemical profiles of four recently developed anti-PSMA mAb to that of 7E11. Specifically, we evaluated these mAbs in prostate cancer cell lines, benign and malignant prostate tissue, benign nonprostate tissue,

and a variety of malignant tissues. In the latter, we sought further to confirm PSMA expression in tumor-associated neovasculature.

MATERIALS AND METHODS

Tissue Specimens and Antibodies. The LNCaP, PC3, and PC3-PIP (PC3 cells transfected with PSMA[4]) were obtained from cell lines cultured in the George M. O'Brien Urology Research Center at Memorial Sloan-Kettering Cancer Center. Fresh-frozen tissue samples from male and female patients were randomly obtained from the Memorial Sloan-Kettering Cancer Center institutional tissue bank. Twenty different benign tissue types, including prostate tissue, were examined, as were the following tumor types: conventional (clear cell) renal cell carcinomas, transitional cell carcinomas of the urinary bladder, testicular-embryonal carcinoma, colonic adenocarcinomas, neuroendocrine carcinomas, glioblastoma multiforme, malignant melanomas, pancreatic duct carcinomas, non-small cell lung carcinomas, soft tissue sarcomas, benign and malignant vascular tumors, breast carcinomas, and prostatic adenocarcinomas. The 7E11 mAb was provided by Cytogen, Inc. (Princeton, NJ). The J591 and J415 antibodies were recently developed, and their characteristics were demonstrated previously (16). The mAbs PEQ226.5 and PM2J004.5 were provided by Hybritech Inc. (San Diego, CA) and also described previously (13). The anti-endothelial cell mAb CD34 (Immunotech, Coulter Company, Opa Locka, FL) was used for comparative immunohistochemical reactions in all cancerous tissue types.

Immunohistochemistry. LNCaP, PC3, and PC3-PIP were grown in cell culture wells to ~80% confluence. Immunohistochemical studies were then performed on the different cell types in either a viable or a fixed state. For fixation, the cells were treated with 10% buffered formalin for 10 min. The cells were then incubated with the different mAbs at 5 µg/ml at room temperature for 45 min. For live cells, after incubation with the primary antibody under the same conditions, the cells were then fixed in cold 10% buffered formalin for 10 min. The immunohistochemical reaction was completed by the streptavidin-biotin method. Briefly, the sections were washed thoroughly in 1.0% PBS, and biotinylated secondary antibody, horse anti-mouse IgG, was added for 60 min. After washing with PBS, streptavidin was added to the specimens for 60 min, and the slides were washed again in PBS. Next, the specimens were immersed for 5 min in a fresh solution of 0.06% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide. Following washing, the sections were counterstained with hematoxylin, dehydrated, and mounted.

Tissue samples were snap-frozen in OCT compound placed in isopentane and stored at -70°C. Multiple 5-µm cryostat tissue sections were then cut and fixed in cold acetone (4°C) for 12 min. Prior to primary mAb incubation, the specimens underwent 30-min incubation with a normal horse blocking serum 20 in 2.0% BSA. The primary antibody incubations (5 µg/ml) were then performed with 7E11, J591, J415, PEQ226.5, PM2J004.5, and CD34 (in the cancer cases) for 60 min at room temperature. The remainder of the immunohistochemical reaction was completed using the streptavidin-biotin method as described previously. In tissue with known significant quantities of endogenous biotin, the immunoperoxidase method was used with rabbit antimouse immunoglobulin-peroxidase as the secondary antibody (Envision; DAKO Corp., Carpinteria, CA). In all tissue sections, negative controls were performed using blocking serum in place of the primary antibody. The immunohistochemical reactivities of all of the mAbs were then evaluated and compared.

RESULTS

Tumor-associated Neovasculature. With rare exceptions, all five anti-PSMA mAbs bound tumor-associated neovasculature of nonprostatic tumors (Table 1 and Fig. 1). The neovasculature of one breast carcinoma and one soft tissue sarcoma (myxofibrosarcoma) showed no immunoreactivity; however, both contained CD34-positive vasculature. The four cases of breast carcinoma with PSMA-positive neovasculature were ductal carcinomas, and the one PSMA-negative case

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Table 1 *Results of PSMA immunohistochemistry in tumor cells and tumor-associated neovasculature*

Tumor	No. of positive tumors/total no. of tumors studied	
	Tumor cells	Neovasculature
Conventional renal cell carcinoma	0/11	11/11
Transitional cell carcinoma	0/6	6/6
Testicular embryonal carcinoma	0/1	1/1
Colonic adenocarcinoma	0/5	5/5
Neuroendocrine carcinoma	0/5	5/5
Glioblastoma multiforme	0/1	1/1
Malignant melanoma	0/5	5/5
Pancreatic duct carcinoma	0/4	4/4
Non-small cell lung carcinoma	0/5	5/5
Soft tissue sarcoma	0/6	5/6
Breast carcinoma	0/6	5/6
Hemangioma	0/3	0/3
Hemangioendothelioma	0/1	0/1
Angiosarcoma	0/1	0/1
Angiolipoma	0/1	0/1
Angiomyolipoma	0/2	0/2
Prostatic adenocarcinoma	12/12	2/12

was lobular carcinoma. Interestingly, only a small subset of prostate cancer specimens showed PSMA-positive neovasculature (2 of 12 cases). In these cases, we found the CD34-stained sections to be useful in localizing so-called "hot spots" of neovasculature that we then compared to the anti-PSMA mAb-stained sections. This helped us confirm the location of vessels amid strongly PSMA-positive tumor cells. We noted no significant histological differences between prostate cancers with PSMA-positive neovasculature and those with PSMA-negative neovasculature. In all of the tumors, 7E11, J591, J415, PEQ226.5, and PM2J004.5 mAbs bound neovasculature in a like manner (Fig. 2). The results of CD34 immunohistochemistry in sequential tissue sections confirmed localization of the anti-PSMA mAbs to neovasculature endothelium (Fig. 2). In contrast to tumor-associated neovasculature, none of the anti-PSMA mAbs reacted with vasculature in the non-cancer-bearing tissue sections. The staining intensity of the external domain-binding mAbs (J591, J415, and PEQ226.5) in tumor-associated neovasculature was greater than that of the internal domain-binding mAbs (7E11 and PM2J004.5).

Malignant Tumor Cells. All 12 prostate cancer cases were strongly PSMA positive, and all nonprostate tumor cells were PSMA negative (Table 1). All vascular tumors were CD34 positive but PSMA negative.

Prostate Cancer Cell Lines. The external domain-binding mAbs (J591, J415, and PEQ226.5) bound viable LNCaP and PC3-PIP cells that are known to express PSMA. In contrast, the internal domain-binding mAbs (7E11 and PM2J004.5) did not bind viable LNCaP and PC3-PIP cells (Fig. 3). After formalin fixation, all anti-PSMA mAbs, including 7E11 and PM2J004.5, reacted with LNCaP and PC3-PIP cells. None of the mAbs bound viable or formalin-fixed PC3 cells that are known to lack PSMA expression.

Benign Tissues. Although benign prostatic secretory-acinar epithelium displayed heterogeneous staining with the five mAbs, all 28 benign prostate cases were PSMA positive. Immunoreactivity was typically concentrated at the luminal aspect of the cytoplasmic membrane. Basal epithelium and stromal cells were PSMA negative. The immunoreactivity of the benign secretory-acinar epithelium was less intense than that of prostatic adenocarcinoma, and the staining intensity of the external domain-binding mAbs J591, J415, and PEQ226.5 was greater than that of the internal domain-binding mAbs 7E11 and PM2J004.5 (data not shown).

The anti-PSMA mAbs reacted with several of the 19 benign non-prostate tissues (Table 2). All five mAbs reacted with duodenal

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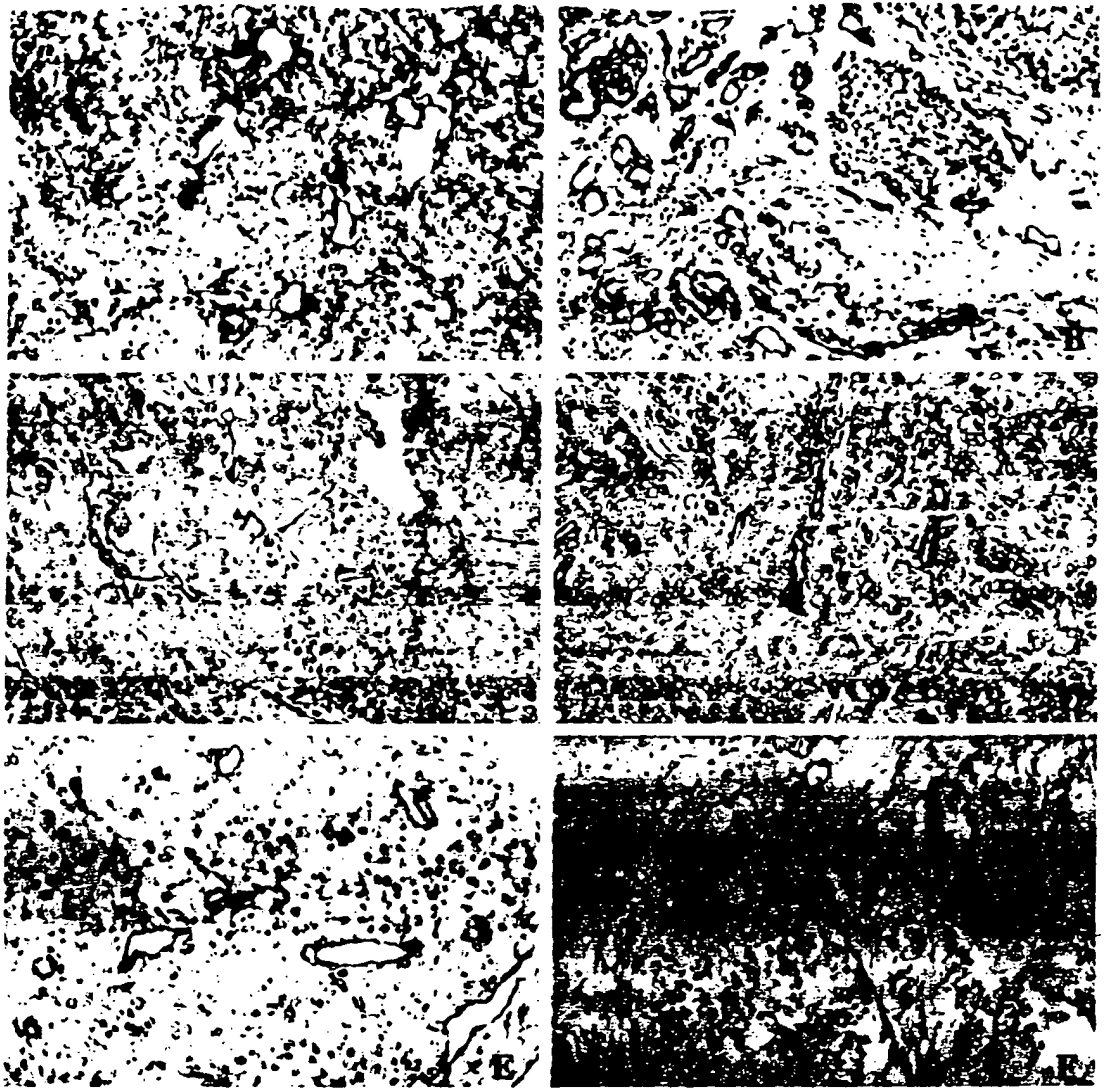[4] J. B. Latouche and M. Sadelain, unpublished observations.

Fig. 1. PSMA expression in tumor-associated neovasculature. Immunohistochemical reactivity with external domain-binding anti-PSMA mAbs J591 or PEQ226.5 in representative cancer types. A, J591, breast cancer; B, PEQ226.5, transitional cell carcinoma of the urinary bladder; C, J591, malignant melanoma; D, PEQ226.5, non-small cell lung carcinoma; E, J591, soft tissue sarcoma; and F, J591, neuroendocrine carcinoma.

columnar (brush border) epithelium (11 of 11 cases), renal proximal tubular epithelium (5 of 5 cases), benign breast epithelium (8 of 8 cases), and colonic ganglion cells (1 of 12 cases). In skeletal muscle, a subset of muscle fibers were positive only with 7E11 and negative with the other four mAbs (Fig. 4). The vasculature in all benign tissues was uniformly PSMA negative. The staining intensity of these PSMA-positive benign tissues was less than that of prostate cancer and tumor-associated neovasculature.

DISCUSSION

Our study confirms PSMA expression in the neovasculature of a wide spectrum of malignant neoplasms. Specifically, we found PSMA expression in various epithelial tumors (carcinomas), neuroendocrine tumors, and mesenchymal tumors (soft tissue sarcomas) and in ma-

lignant melanoma and glioma. In contrast to previous studies, we used five anti-PSMA mAbs, each of which binds a different epitope of the intracellular or extracellular PSMA domain. Thus, our results provide further evidence that PSMA, rather than a PSMA-like molecule, is expressed in tumor-associated neovasculature. Also in contrast to previous studies, we confirmed localization of PSMA to endothelial cells with the mAb CD34, an anti-endothelial cell marker used to study angiogenesis and determine microvessel density (26–30).

Our findings are consistent with previous studies showing PSMA expression in tumor-associated neovasculature. For example. Silver et al. (15) demonstrated 7E11 binding and "neoexpression of PSMA in endothelial cells" in a subset of tumors, including renal cell carcinoma (unspecified type), transitional cell carcinoma of the urinary bladder, and colonic adenocarcinoma. More recently, Liu et al. (16) studied
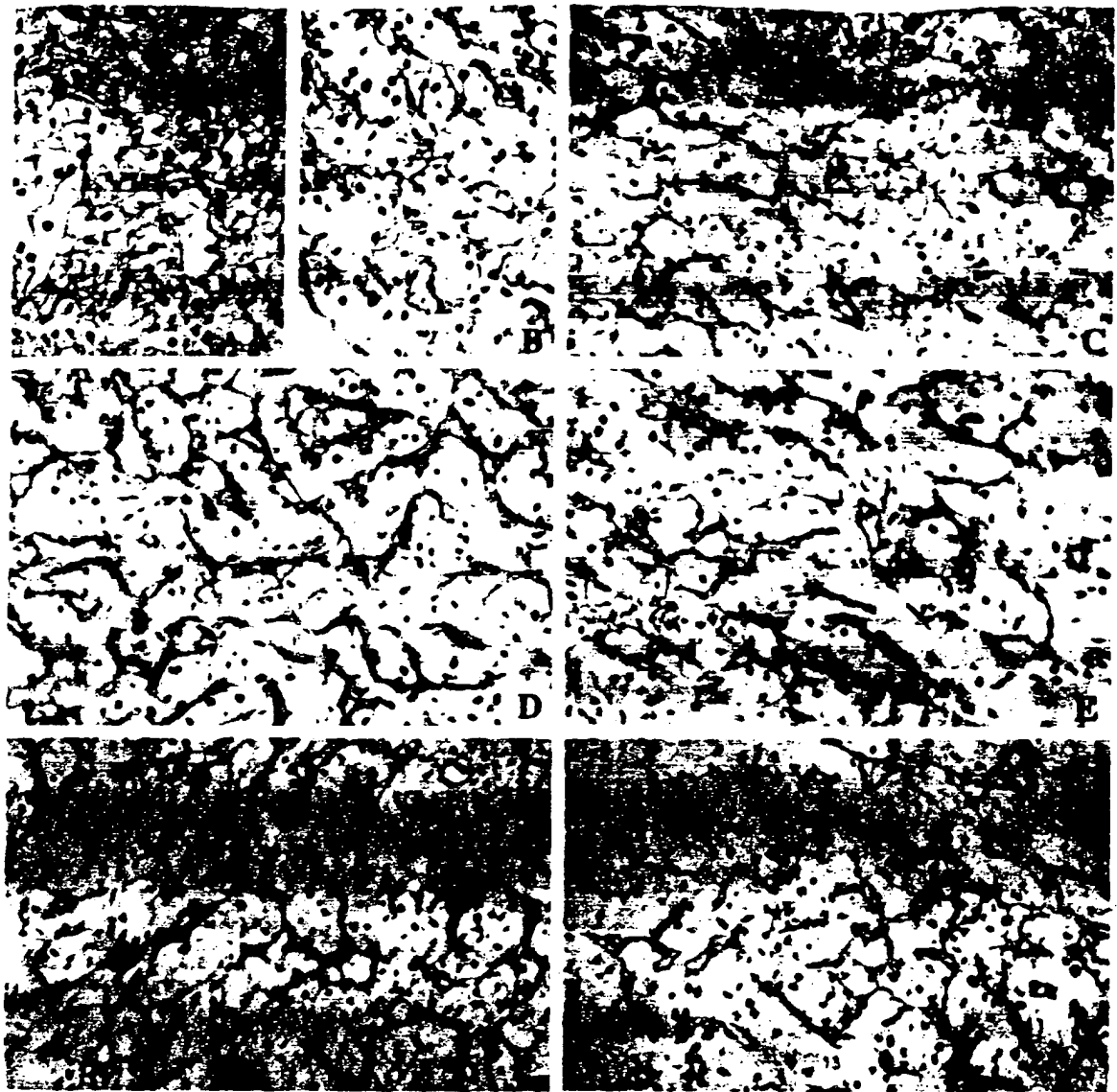
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Fig. 2. Binding profile of the five anti-PSMA mAbs and CD34 in the neovasculature of conventional (clear cell) renal cell carcinoma. A, H&E-stained section; B, CD34; C, 7E11; D, J591; E, J415; F, PEQ226.5; and G, PM2J004.5.

four external domain-binding anti-PSMA mAbs (J591, J415, J533, and E99) and showed that each bound the tumor-associated neovasculature in several nonprostatic carcinomas. Although it is unclear whether PSMA is produced by endothelial cells of tumor-associated neovasculature or whether it is produced in other tissues and sequestered from the serum, we favor the former because PSMA is expressed only in a limited number of benign tissues and in prostate cancer but is not expressed in other malignant cell type. In addition, circulating PSMA has not been demonstrated in serum.[5] Additional studies, however, are necessary to confirm this hypothesis.

We found that endothelial cell expression of PSMA was restricted

to the neovasculature of malignant neoplasms. In fact, neither the vascular endothelial cells of benign tissues nor the neoplastic cells of vascular tumors expressed PSMA. These results suggest that endothelial cell-PSMA expression may be stimulated by one or more tumor-secreted angiogenic factors. The fact that all of the vascular neoplasms we studied, including the one example of angiosarcoma, were PSMA negative is not surprising, given that, in these tumors, the endothelium itself is neoplastic and, presumably, not stimulated by angiogenic factors. The presence or absence of PSMA expression in benign neovasculature (e.g., granulation tissue, endometrium, and so on) remains to be established.

The neovasculature associated with OC prostatic adenocarcinoma only rarely expressed PSMA. Others also have found no detectable

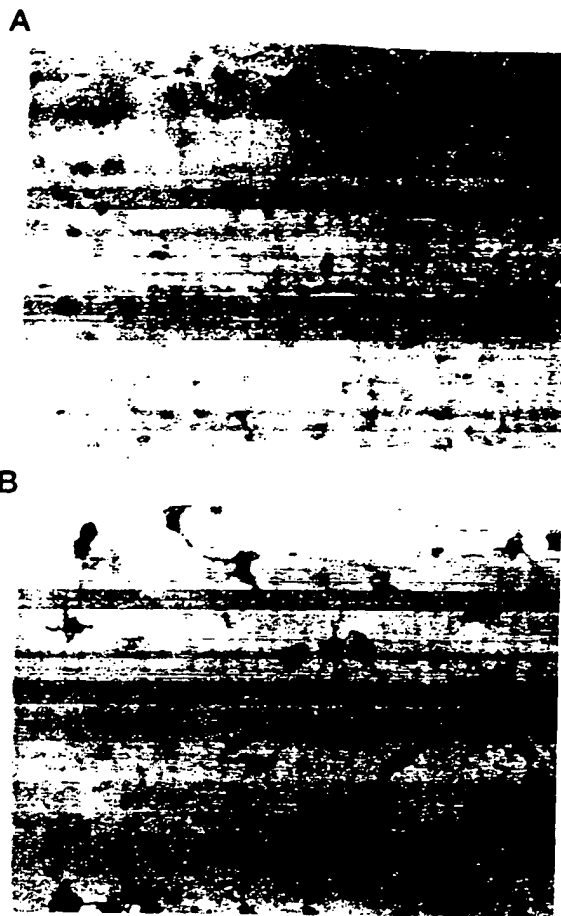[5] H. Liu and N. H. Bander, unpublished observations.

A

B

Fig. 3. Comparative immunohistochemistry in viable PSMA-expressing PC3-PIP cells. A. 7E11 demonstrating no immunoreactivity. B. J591 demonstrating positive immunoreactivity with live cells.

PSMA expression in OC prostate cancer-associated neovasculature (9, 15). These observations are remarkable given the ubiquity of PSMA expression in tumor-associated neovasculature of other cancer types. They are. however, not altogether surprising, given the histological features of OC prostate cancer. For example, in contrast to many other epithelial tumors such as ductal carcinoma of the breast or pancreas, OC prostate cancer typically is not associated with an exuberant host-stromal reaction. Lobular carcinoma of the breast, like prostatic adenocarcinoma. typically does not induce a marked desmoplastic stromal response. Interestingly, the one breast cancer specimen in our series with PSMA-negative neovasculature was an example of lobular carcinoma. These results suggest that PSMA expression in tumor-associated neovasculature may be related to the degree and nature of neoangiogenesis. The relationship between primary tumor stage in different malignancies and PSMA expression in neovasculature is unknown.

Consistent with most previous studies. we found that mAbs to the intracellular PSMA domain (7E11 and PM2J004.5) do not bind viable prostate cancer cells, whereas mAbs to the external domain (J591, J415, and PEQ226.5) do bind live cells (16, 21). Only one study has reported 7E11 binding with viable prostate cancer cells (31). It is

postulated that 7E11 binds predominantly to apoptotic cells within prostate cancer sites in vivo. Apoptotic cells, unfortunately, comprise only a minority of the total prostate tumor-cell population. This. no doubt. has contributed to the relatively low sensitivity of ^{111}In-capromab pendetide as an imaging agent for prostate cancer. In this regard. targeting the extracellular PSMA domain with radioimmuno-conjugates may enhance prostate cancer cell labeling in vivo.

The results of several but not all immunohistochemical studies using the 7E11 mAb have shown that PSMA is expressed in a limited number of nonprostatic tissues (1. 6. 15). Our findings support the results of other studies showing PSMA expression in duodenal (brush border) epithelium and renal proximal tubular epithelium but suggest that PSMA expression in these tissues is less than it is in prostate cancer and tumor-associated neovasculature (15. 16). Duodenal brush-border epithelium has high levels of folate hydrolase activity that is essential for folate absorption (17). This folate hydrolase activity is localized to the luminal membrane and is consistent with the staining pattern of the anti-PSMA mAbs. Proximal renal tubular epithelium also actively reabsorbs folate through the luminal membrane (32). Halsted et al. (33) found significant sequence homology between pig intestinal folate hydrolase (folypoly-gamma-glutamate carboxypeptidase) and human PSMA. suggesting that human duodenal membrane folate hydrolase may represent PSMA. Alternatively. it may represent a closely related enzyme that cross-reacts with anti-PSMA mAbs. In contrast to previous studies. we found consistent PSMA expression in mammary ductal epithelium. The reasons for our conflicting results are unclear: however. previous studies showing no PSMA expression in breast may have included specimens with inadequate amounts of ductal epithelium. One of our 12 colon specimens displayed PSMA expression in ganglion cells. The relatively sparse immunoreactivity observed in colonic ganglia may be indicative of peripheral neuronal PSMA expression previously described in nonmyelinating, perisynaptic Schwann cells near motoneuron terminal endplates (34).

The staining profile of skeletal muscle is unique, in that a subset of cells is positive with only 7E11. Liu et al. (16) also showed a subset of skeletal muscle cells bind 7E11 and not other anti-PSMA mAbs. Of

Table 2 Results of PSMA immunohistochemistry using five different anti-PSMA mAbs in fresh-frozen benign tissue

Tissue	No. of positive cases/total no. of cases studied				
	7E11	J591	J415	PEQ226.5	PM2J004.5
Prostate	28/28	28/28	28/28	28/28	28/28
Lung	0/5	0/5	0/5	0/5	0/5
Brain	0/3	0/3	0/3	0/3	0/3
Digestive system					
Parotid	0/5	0/5	0/5	0/5	0/5
Esophagus	0/4	0/4	0/4	0/4	0/4
Stomach	0/6	0/6	0/6	0/6	0/6
Duodenum	11/11	11/11	11/11	11/11	11/11
Ileum	0/2	0/2	0/2	0/2	0/2
Colon	1/12	1/12	1/12	1/12	1/12
Pancreas	0/7	0/7	0/7	0/7	0/7
Liver	0/5	0/5	0/5	0/5	0/5
Genitourinary system					
Kidney					
Glomeruli	0/5	0/5	0/5	0/5	0/5
Proximal tubules	5/5	5/5	5/5	5/5	5/5
Distal tubules	0/5	0/5	0/5	0/5	0/5
Collecting ducts	0/5	0/5	0/5	0/5	0/5
Bladder	0/5	0/5	0/5	0/5	0/5
Testis	0/9	0/9	0/9	0/9	0/9
Breast	8/8	8/8	8/8	8/8	8/8
Ovary	0/5	0/5	0/5	0/5	0/5
Skin	0/5	0/5	0/5	0/5	0/5
Skeletal muscle	7/7	0/7	0/7	0/7	0/7
Endocrine system					
Thyroid	0/5	0/5	0/5	0/5	0/5
Adrenal cortex/medulla	0/5	0/5	0/5	0/5	0/5

Fig. 4. Skeletal muscle. *A*, H&E-stained section. *B*, 7E11 immunohistochemical stain showing positive reaction in a subset of cells. *C*, PM2J004.5 immunohistochemical stain showing no reactivity.

plasms. The importance of angiogenesis in neoplasia is well documented (38–40), and endothelial cell expression of PSMA appears highly restricted to tumor-associated neovasculature and may represent a novel target for antineovasculature based therapy. Recent *in vivo* localization by the ^{111}In-labeled 7E11 mAb to a conventional (clear cell) renal cell carcinoma demonstrates the potential clinical utility of anti-PSMA mAbs in a nonprostate cancer (41). Enthusiasm for mAb-based therapy, however, must be tempered by the fact that PSMA is expressed in several benign tissue types; the potential side effects of anti-PSMA mAbs on these tissues *in vivo* is unknown. However, other mAbs that are currently in clinical trials or Food and Drug Administration-approved for clinical use, also are not tumor specific and bind antigens expressed in benign tissues (42, 43).

note is the fact that the other internal domain-binding anti-PSMA mAb, PM2J004.5, did not bind skeletal muscle. Thus, it is likely that, in skeletal muscle, 7E11 uniquely cross-reacts with either a yet to be defined PSMA-like or a PSMA-unrelated molecule. The patchy distribution suggests that expression of this molecule may be restricted to either fast-twitch or slow-twitch muscle fibers.

Novel PSMA-based prostate cancer therapies, including anti-PSMA mAb-based therapies, are currently under investigation (35–37). The results of our study indicate that anti-PSMA mAb-based diagnostic and therapeutic modalities may be expanded to include antineovasculature targeting for a wide variety of malignant neo-

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produced 25.3% and 64.9% lysis of SKOV3 cells, respectively. The B1D2 x NM3E2 scFv₂ promoted cytotoxicity at concentrations as low as 100pg/ml. Thus, high affinity binding to tumor antigens increases bispecific scFv₂-mediated cytotoxicity, possibly because the slow off-rate of the higher affinity bispecific scFv₂ prolongs its cell surface retention. Additional bispecific constructs are currently being tested to further examine the role of affinity over a 10,000-fold range of K_D values.

#2993 Antibody-TNF fusion constructs targeting HER2 can overcome HER2-mediated resistance to TNF. Rosenblum, M., Parakh, C., Hom, S., and Cheung, L. *University of Texas—M.D. Anderson Cancer Center, Houston, TX 77030.*

Overexpression of the proto-oncogene HER2/NEU in breast cancer and certain other tumors appears to be a central mechanism which may be partly responsible for cellular progression of the neoplastic phenotype. Transfection studies with HER2/NEU results in reduced sensitivity to TNF's cytotoxic effects and reduced sensitivity to immune effector killing. The single-chain recombinant antibody sfv23 recognizes the cell-surface domain of HER2. The cDNA for this antibody was fused to the cDNA encoding human TNF and this fusion construct was cloned into a plasmid for expression in E. coli. The fusion protein was expressed as insoluble inclusion bodies and renatured after solubilization in 6M guanidine and purified by ion exchange chromatography. SDS-PAGE demonstrated a single band at the expected molecular weight (43 kDa). The fusion construct was tested for TNF activity against L-929 cells and found to have TNF activity (S.A. 420 nM). The construct was also tested by ELISA for binding against SKBR-3 (HER2 positive) cells. Cytotoxicity studies against SKBR-3 cells demonstrate that the sfv23/TNF fusion construct was 500 fold more active than free TNF and therefore apparently able to overcome the HER-2 mediated resistance to TNF. Further in vitro studies to examine the biological properties of this agent are ongoing.

#2994 Characterization of Anti-HER-2 Monoclonal Antibodies Which Inhibit the Growth of Breast Cancer Cell Lines. Ilgen, A., Ghetie, M., Shen, G., Li, J., Uhr, J. and E. Vitetta. *Cancer Immunobiology Center, UT Southwestern Medical Center.*

HER-2, or c-erbB-2, is a member of the EGF receptor family. Overexpression of the wild type HER-2 protein, as is observed on numerous carcinomas, leads to hyperactivity of the kinase and confers a significant growth advantage on cells. Numerous groups have generated monoclonal antibodies (MAbs) against HER-2 which inhibit the growth of breast cancer cell lines. Our research had three goals: to understand the mechanisms by which anti-HER-2 MAbs inhibit the growth of breast cancer cells, how the physical properties of the MAbs related to their mechanism of inhibition, and how we can optimize the anti-tumor activity of these MAbs. We generated a panel of 113 Mabs against HER-2 in this manner. 12 of which inhibited the growth of a panel of HER-2-overexpressing breast cancer cells. To understand the mechanism of inhibition of growth, we determined whether the MAbs induced cell cycle arrest and/or apoptosis in treated cells. We found that each of these 12 MAbs signaled the cells to undergo varying degrees of apoptosis and/or cell cycle arrest and that the signaling capabilities of the Mabs correlated with both the extent of overexpression of HER-2 on the breast cancer cell lines and the affinity of the MAbs. Our next step will be to determine whether MAbs and immunotoxins against different epitopes on the HER-2 molecule are able to synergistically inhibit the growth of the breast cancer cells.

#2995 Anti-metastatic therapy of MDR human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction in SCID mice. Nokihara, H., Hanibuchi, M., Yanagawa, H., Shinohara, T., Fujiki, F., Nishinuraa, N., Parajuli, P., Tsuruo, T., and Sone, S. *Third Department of Internal Medicine, Tokushima University School of Medicine, Tokushima 770, Japan. and Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113, Japan.*

Distant metastasis is a critical problem in the therapy of human lung cancer. In this study, we investigated whether transduction of the monocyte chemoattractant protein-1 (MCP-1)-gene into multidrug-resistant (MDR) human lung cancer cells affected the inhibition of their metastases by the anti-P-glycoprotein (P-gp) monoclonal antibody MRK16. MDR human small cell lung cancer (SCLC), H69/VP cells, were transduced with human MCP-1-gene inserted into an expression vector (BCMGSNeo). MCP-1-gene transduction had no effect on the expression of P-gp, drug sensitivity to etoposide or the in vitro proliferation. In the metastatic model of NK-cell depleted SCID mice, the number of metastatic colonies of MCP-1-gene transduced H69/VP cells were similar to those of parent or mock-transduced cells. However, systemic treatment with MRK16 was more effective in inhibiting the metastasis of MCP-1-producing H69/VP than mock-transduced cells. These findings suggest that local production of MCP-1 in tumor site may increase the anti-P-gp antibody dependent cell-mediated cytotoxicity. This can be a useful immunological strategy to inhibit the metastasis of MDR human lung cancer cells expressing P-gp.

#2996 Alpha particle emitter therapy of micrometastases: ²¹³Bi-J5, (anti-PSMA) treatment of LNCaP spheroids. Yang, W-H., Ballangrud, A., McDevitt, M.R., Finn, R.A., Geerlings, M., Bander N., Scheinberg, D.A., Sgouros, G. *Memorial Sloan-Kettering Cancer Center, NY, NY 10021. PharmaCtinium, Inc. Wilmington, DE 19801, Cornell University Medical Center, NY 10021.*

Multicellular spheroids were used to investigate the feasibility of eradicating micrometastases with radiolabeled antibodies. Spheroids of LNCaP (LN3) were exposed to 2 concentrations of bismuth-213-labeled J591 antibody (50 and 100 μCi) for 4 different incubation durations (15,30,60 min and 18 h). J591 targets an extracellular epitope of the prostate-specific membrane antigen (PSMA). ²¹³ emits alphas of 60 to 90 μm range: it has a 45.6 min. half-life. 24 spheroids, of 100 to 200 μm diameter were used for each experiment. Growth curves were obtained for each spheroid up to day 70, post-incubation. ²¹³Bi-HuM195 (anti-CD33), was used as a "hot" control. Spheroids exposed to 100 μCi ²¹³Bi-J591 for more than 15 min. did not re-grow; controls showed growth delay but re-grew except following an 18 h incubation. The growth of spheroids exposed to 50 ²¹³Bi-J591 was arrested only after an 18 h exposure; at days 33 and 68, median volume ratios of 400 and 7400 between hot control and specific antibody were obtained. The results demonstrate feasibility and efficacy in using antibodies labeled with alpha particle emitting radionuclides to target small cluster of tumor cells or micrometastases. The results obtained using this spheroid model may be used, in combination, with mathematical modeling to evaluate different treatment protocols against micrometastases and to optimize such a treatment approach.

#2997 A novel humanised antibody against Prostate Specific Membrane Antigen (PSMA) for in vivo targeting and therapy. Hamilton, A., King, S., Liu, Moy, P., Bander, N., and Carr, F. *Biovation Ltd, Aberdeen AB24 3RY, Department of Urology, The New York Hospital-Cornell Medical Center, New York, NY 10021, Ludwig Institute for Cancer Research, New York Branch, New York, NY 10021.*

A murine monoclonal antibody (mAb), J591, is directed the against the extracellular domain of PSMA, an integral membrane protein of prostate carcinoma and of tumour vascular endothelium of a wide variety of cancers, but not normal endothelium. The mAb was "humanised" by a novel method involving specific deletion of human B and T cell epitopes. To remove B cell epitope surface exposed residues in the frameworks of the murine J591 heavy and light chain variable region (V_H and V_L) sequences were substituted by the corresponding residues from selected human germ-line V_H and V_L sequences ("surface humanisation"). For detection and elimination of T cell epitopes, a database human MHC class II binding peptides was searched for motifs present in substituted V_H and V_L sequences and in addition a novel computer modelling approach termed "peptide threading" was applied. Motifs, unless also present human germ-line antibody sequences, were deleted by substituting single amino acids, preserving the CDRs. The final sequences were re-checked for new MHC class II motifs. The designed V_H and V_L regions were constructed by mutagenesis of the murine V_H and V_L. Human IgG1 or κ constant regions were added and composite genes transfected into NS0 cells to produce complete recombinant antibodies. These antibodies bound to PSMA (on LNCaP cells) as efficiently as original murine antibody, and are expected have little or no immunogenicity man.

#2998 Serum TA90 immune complex correlates with recurrence following adjuvant immunotherapy for regional metastatic melanoma. Hsuen, E.C., Gupta, R.K., Yee, R., Leopoldo, Z., Qi, K., and Morton, D.L. *John Wayne Cancer Institute, Santa Monica, CA 90404.*

We previously reported a significant correlation between clinical evidence melanoma and the presence of a circulating immune complex (IC) composed a 90-kD tumor-associated antigen (TA90) and anti-TA90 antibody. In the present study we hypothesized a correlation between TA90-IC and clinical recurrence melanoma following lymphadenectomy and postoperative adjuvant immunotherapy. We studied 100 melanoma patients who had undergone resection of nodal metastases and postoperative adjuvant therapy with a polyvalent melanoma cell vaccine (PMCV), and from whom serum samples had been obtained after surgical resection but prior to initiation of vaccine therapy. These serum specimens were retrieved from cryopreserved storage, coded, and tested in a blinded manner for TA90-IC. Median follow-up was 25 months (range, 18–78 months). By univariate analysis with log rank test, a positive TA90-IC level was highly correlated with recurrence. Median disease-free survival and 3-year disease-free survival rate were 8 months and 12%, respectively, for the 50 patients with a positive TA90 level, compared with >25 months and >53%, respectively, for the 50 patients with a negative TA90 level (p=0.0001). Multivariate analysis with Cox proportional hazard regression considered TA90, number of positive lymph nodes, size involved lymph nodes, extranodal extension, and disease-free interval. TA90 positivity was the most significant independent variable correlating with disease-free survival (p=0.0001). These data indicate that the presence of TA90-IC in patients with no clinical evidence of melanoma postoperatively is highly correlated with subsequent disease recurrence.

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RAPID COMMUNICATION

Measurement of Prostate-Specific Membrane Antigen in the Serum With a New Antibody

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ABSTRACT: Work to date has identified prostate-specific membrane antigen (PSMA) as a membrane-bound glycoprotein with high specificity for prostatic epithelial cells. PSMA reacts with the monoclonal antibody 7E11.C5, which is present in serum, seminal fluid, and prostatic epithelial cells, and is increased in its expression in the presence of a hormone refractory state associated with prostatic cancer. This report confirms these results and further documents the presence of the monoclonal antibody 3F5.4G6, which reacts with the extracellular domain of PSMA. This region of PSMA is also an element present in a truncated version of the protein, so-called PSM'. Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species that are reactive with the reciprocal antibody in Western blot analysis. Thus, 3F5.4G6 recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. These two antibodies are well suited for use in a sandwich immunoassay, either one as a capture or detection antibody. Current work on this is underway.

This report also confirms that 7E11.C5 Western blots for PSMA are negative with normal human brain tissue. The monoclonal antibody 9H10 does not react with 3F5.4G6 or with 7E11.C5 in studies conducted herein. Moreover, 3F5.4G6 reacts with PSMA found in the LNCaP cell line, but not DU-145 or PC3, which lack PSMA.   © 1996 Wiley-Liss, Inc.

KEY WORDS: prostate-specific membrane antigen (PSMA), prostate cancer, prostate marker

INTRODUCTION

We have previously described an antibody 7E11.C5 that can, by enzyme-linked immunosorbent assay (ELISA) (with another antibody 9H10) or by Western blot, detect in prostate cancer patients a protein called prostate-specific membrane antigen (PSMA) [1–3]. To validate this observation further, we collected sera in a prospective multicenter study. The sera were run on a double-blind basis without knowledge of the clinical state, or the presence or absence of prostate cancer [4]. Furthermore, we compared levels of prostate-specific antigen (PSA) concurrently with PSMA values [4]. The elevated PSMA levels predicted a state of clinical progression or clin-ical resistance in most cases (>70%). PSMA levels were of better prognostic value than PSA [4]. Despite these observations, there have recently been publications questioning whether PSMA was present in the serum [5,6]. We have completed a series of experiments which validate our original observations, and herein report such.

MATERIALS AND METHODS

Cell Lines and Reagents

P3X63Ag8U.1 (X63), an HPGRT-negative mouse myeloma cell line (CRL 1597 from ATCC, Rockville, MD), was maintained in 90% RPMI-1640: 10% Fetal Clone (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, penicillin/streptomycin, and 1 mM sodium pyruvate. Rabbit antimouse IgM and antimouse IgG were purchased from ICN (Costa Mesa, CA). Peroxidase-labeled goat antimouse IgG and goat antimouse IgM were purchased from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, MD). LN-CaP, a prostate cancer cell line (CRL 1740, from ATCC, Rockville, MD) expressing PSMA, was maintained in 95% RPMI-1640/5% fetal calf serum (FCS).

Preparation of Immunogen and Immunization of Mice

PSMA-derived peptide 716–723 (NH$_2$-ESKVD-PSK−) was coupled to keyhole limpet hemocyanin (KLH) as a carrier protein using the EDC coupling method of Pierce (Rockford, IL). The peptide–carrier complex was emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO) containing 1 mg/ml muramyl dipeptide (MDP, Pierce) at a final concentration of 250 μg/ml. BALB/c mice were immunized subcutaneously with 100 μl of the emulsified peptide–carrier complex every 2 weeks. Following the third injection, blood was obtained from the mice and their sera were tested for antipeptide antibodies in a peptide-specific radioimmunoassay (RIA). Spleens from donor mice demonstrating an antipeptide titer of 1:1,000 or greater were used in a fusion protocol with X63 myeloma cells.

Fusion Protocol and Initial Screening for Antipeptide Producing Hybridomas

Three days prior to fusion, the donor mouse was immunized intraperitoneally with 50 μg of peptide–carrier complex in saline. The spleen was aseptically removed, and a single cell suspension was prepared in RPMI-1640 medium without serum. The splenocytes were added to X63 myeloma cells at a ratio of 10:1, and the fusion was performed by the method of Galfré and Milstein [7]. Following fusion, the splenocyte–myeloma mixture was resuspended in 80% RPMI-1640/20% Fetal Clone supplemented with aminopterin, to act as a selective medium for hybridoma growth and plated in 200-μl volumes into sterile 96-well microtiter plates.

Ten to 14 days following fusion, 50 μl of cell culture supernatant from the individual wells was removed and tested in an RIA for peptide-specific antibodies. Briefly, the supernatants were added to

wells of a Pro-Bind plate (Falcon) coated with PSMA-peptide coupled to bovine serum albumin (BSA) by the EDC method referenced above and blocked with BSA. Following an overnight incubation at 4°C the plates were washed 4 times with PBS-0.1% BSA. Fifty μl of a 1:500 dilution of rabbit antimouse IgM and antimouse IgG was added to each well, and the plates were incubated for 1 hr at room temperature. Following four washes, 50 μl of ^{125}I-Protein A ($\approx$10^5 cpm/well) was added to each well and the plates incubated for an additional hour at room temperature. The plates were washed four times and exposed to X-ray film (Kodak, X-OMAT) overnight and developed. Positive wells showing antipeptide-specific antibodies were identified from the exposed film and the cells from the positive wells were expanded in 90% RPMI-1640/10% Fetal Clone for further testing.

Western Blot Analysis of Primary Hybridomas for anti-PSMA Antibody

Supernatants from the expanded antipeptide-reactive primary hybridomas were tested in a Western blot assay for the presence of anti-PSMA antibodies. Western blot analysis was performed following the protocol of Pelletier and Boynton [8]. Briefly, lysates from LNCaP cells, a prostatic tumor cell line that expresses PSMA, were electrophoresed on an 8.5% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electroblotted onto a PVDF membrane for 1 hr at 90 V. The membranes were blocked overnight in 5% milk–TBS. The blocked membrane was placed in a multiscreen apparatus (BioRad), and approximately 650 μl of hybridoma supernatant was pipetted into individual lanes. Following a 90 minute incubation at room temperature the blot was removed from the apparatus, washed five times for 5 min in TBS–0.5% Tween-20 (TBS-T), and probed with 167 ng/ml of peroxidase-labeled goat antimouse IgG secondary antibody (KPL, Gaithersburg, MD) for 30 min at room temperature. The membrane was washed five times for 5 minutes with TBS-T, and the membrane was developed using the Chemiluminescent Substrate Kit (KPL). The blot was visualized by exposing X-ray film. Positive hybridomas (anti-PSMA reactivity) were identified and selected for further development.

Cloning by Limiting Dilution, Testing Clones, and Purification of Monoclonal Antibody

Primary hybridomas identified by their anti-PSMA reactivity were cloned by limiting dilution. Briefly, the cells were adjusted to a final concentration of 1 cell/ml of RPMI-1640–10% Fetal Clone containing 10^5 syngeneic thymocytes as a feeder cell population. Two hundred μl of cell suspension was pipetted into wells of

sterile 96-well microtiter plates and cultured for 7–10 days, or until single colonies of cells were visible. Wells containing single colonies were picked, and the clones were expanded in 24-well plates. Supernatants from the clonal cultures were harvested and tested for anti-PSMA reactivity in the Western blot assay described above. Clones producing anti-PSMA monoclonal antibody were expanded, and the cells were used for the generation of high titer ascites fluid. The monoclonal antibody 3F5.4G6, an IgM class anti-PSMA antibody, was purified from ascites fluid using the ImmunoPure IgM Purification Kit (Pierce, Rockford, IL).

Immunoprecipitation of PSMA from LNCaP Tumor Cells Using 3F5.4G6 Monoclonal Antibody

Approximately 10×10^6 LNCaP tumor cells were incubated with 1 ml of NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) for 30 min at 4°C. The lysate was centrifuged at 12,000 rpm for 5 min to remove cellular debris and the resultant supernatant was precleared by incubation with 50 μl of normal mouse serum (Sigma) for 30 min followed by the addition of 60 μl of a 20% suspension of antimouse IgM-coupled agarose beads (Sigma). Following 1-hr incubation at 4°C, the supernatant was centrifuged at 12,000 rpm to remove the beads, the resultant supernatant was used in an immunoprecipitation protocol with 3F5.4G6 monoclonal antibody. Ten μg of purified 3F5.4G6 monoclonal antibody was added to the supernatant and incubated for 1 hr at 4°C. One hundred μl of a 10% suspension of antimouse IgM-coupled agarose beads was added, and the supernatant was incubated for an additional hour at 4°C. The samples were centrifuged at 12,000 rpm, and the agarose beads were washed three times with NP-40 lysis buffer. The agarose beads were resuspended in 30 μl of sample buffer (SDS reducing buffer) and heated for 10 min at 95°C. Following a brief centrifugation at 12,000 rpm, the sample was run on an 8.5% SDS–PAGE gel, and the separated proteins were electroblotted as described above. A Western blot assay as described above was performed on the samples using the PSMA-specific monoclonal antibody 7E11.C5 as the reporting antibody.

In additional controlled studies, the 9H10 monoclonal antibody was employed. This antibody reacts with an unknown protein on the surface of only LN-CaP cells as was initially described, in contrast to 7E11.C5, which reacts to prostate epithelial cells [3].

RESULTS AND DISCUSSION

PSMA is a membrane-bound glycoprotein that shows high tissue specificity for prostatic tissues.
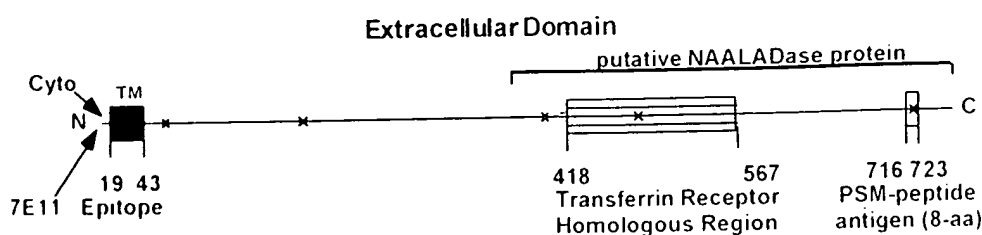

Fig. 1.   Western blot assay of LNCaP lysate using 7E11.C.5 (lane 1) and 3F5.4G6 (lane 2) monoclonal antibodies and developed with HRP-anti-IgM secondary antibody. It should be noted that 3F5.4G6 recognizes a protein of M_r 120 kDa, which is similar, if not identical, to the protein recognized by 7E11.C5. 3F5.4G6 monoclonal antibody also recognizes a protein of M_r 110 kDa corresponding to the protein PSM'. It should be noted that 7E11.C5 does not recognizePSM' because the epitope of 7E11.C5 monoclonal antibody is N-terminal (amino acid 1'7) and is not found in PSM' because PSM' is a truncated version of PSMA and does not contain the initial 57 amino acids of PSMA. Thus, 3F5.4G6 recognizes the C terminal portion of the protein (amino acid 716–723) and specifically the extracellular domain of PSMA and PSM'.

PSMA was originally defined and its tissue expression characterized based upon the reactivity of the monoclonal antibody 7E11.C5 [3]. These results indicated high specificity for prostatic tissues and an increased expression of the 7E11.C5 antibody in the serum of prostatic cancer patients compared to normal individuals [2,3]. Further studies have confirmed this observation [1,4]. Other studies suggest that PSMA expression in tumors is down-regulated by steroids such as 5α-dihydrotestosterone [9,10]. This behavior is consistent with the elevated expression of PSMA in hormone-refractory tumors. Thus, the results indicate the antigen are almost entirely prostate specific in humans; furthermore, they may be a marker for aggressive clones of prostate cancer cells due to its increased expression in hormone-resistant tumors [1–4]. Figure 1 illustrates on Western blot the reactivity of 7E11.C5 with PSMA in LNCaP cells. It should be noted that monoclonal antibody 9H10 does not recognize a protein (i.e., PSMA) of M_r 110–120 kDa in LNCaP cells, but rather recognizes a protein of M_r 30 kDa of unknown identity.

Using the 7E11.C5 antibody as a probe, Israeli et al. [9] cloned a cDNA from LNCaP cells that encodes a 750-amino acid membrane protein. The deduced amino acid sequence defines a type II transmembrane protein composed of a short cytoplasmic domain, a membrane-spanning domain, and an extracellular domain, a portion of which has high sequence homology to transferrin receptor [9]. Further work has shown that the protein epitope to which the 7E11.C5 antibody binds is composed of the N-terminal amino acids of the protein located in the cytoplasmic domain [5,6]. That is, a "sixmer" composed of the first six amino acids from the N-terminal of PSMA was the

PROSTATE SPECIFIC MEMBRANE ANTIGEN

Extracellular Domain

putative NAALADase protein

Cyto TM

N C

19 43
7E11 Epitope

418 567
Transferrin Receptor
Homologous Region

716 723
PSM-peptide
antigen (8-aa)

HOPP & WOODS ANTIGEN PREDICTION

AMINO ACID POSITION	SEQUENCE
479 - 486	K E L K S P D E
404 - 414	K K E G W R P R R
183 - 191	K E L K S P D E G
63 - 69	D E L K A E N
716 - 723	E S K V D P S K

Fig. 2. Topographical representation of PSMA regions in relation to Hopp and Woods antigen prediction. A recent paper reported a rat brain partial cDNA clone of NAALADase which possesses a high degree of identity to the 3' end of the PSMA cDNA. The putative NAALADase protein is highly homologous to a region of the extracellular domain of PSMA.

smallest peptide element recognized by 7E11.C5. No binding to any other peptide element not containing this terminal sequence was reported [5,6].

A variant of PSMA resulting from alternative splicing has been deduced from reverse transcriptase polymerase chain reaction (RT-PCR) studies and RNase protection assays [9]. This variant, designated PSM', is missing the first 57 amino acids of PSMA and was reported to predominate over PSMA in normal prostatic tissues while the reverse relationship was true in the case of prostatic carcinomas [10]. Thus, the PSM' protein is not recognized by the 7E11.C5 antibody (Fig. 1), and data supporting the physiological expression of PSM' to date rely solely on studies of the nature of mRNA species expressed in prostatic cells and tissues.

We have been interested in expression of PSMA as a possible marker for disease progression, particularly in later stages of the disease. Efforts have so far focused on development of serodiagnostic assays relying on the 7E11.C5 antibody for detection in a quantitative Western blot assay with serum. We now report, herein, the preparation of a monoclonal antibody designated 3F5.4G6 that is specific for an 8-amino acid region located near the C-terminal portion of the molecule (aa 716–723). An analysis of antigenic determinants based upon the Hopp and Woods algorithm demonstrated the antigenic potential of this protein region [11] (Fig. 2). Its presence in

the extracellular domain near the C-terminal of the protein makes this determinant ideal for application to a sandwich-type immunoassay or to detect the presence of PSM' in the serum.

The results presented indicate that the 3F5.4G6 monoclonal antibody reacts specifically with the same protein species as 7E11.C5 in Western blots with LN-CaP cell crude lysates (Fig. 3). Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species, which in both cases is reactive with the reciprocal antibody in Western blot analyses (Fig. 3). Thus, it can be concluded that the 3F5.4G6 antibody recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. The 3F5.4G6 antibody is thus well suited for use in a sandwich immunoassay as either a capture or detection antibody paired with the 7E11.C5 antibody. Current work is focused on this strategy.

The 3F5.4G6 antibody, in addition to its binding to the PSMA protein, cross-reacted with a protein species which correlates in size with the PSM' variant present in Western blots of LNCaP cell lysates (Fig. 3) by virtue of its specificity for a common epitope in the extracellular domain of both protein forms [10]. This, for the first time, provides direct evidence of the expression of PSM' at the protein level and provides a possible means for differential quantitation of each protein form in serum. 3F5.4G6 antibody also recog-
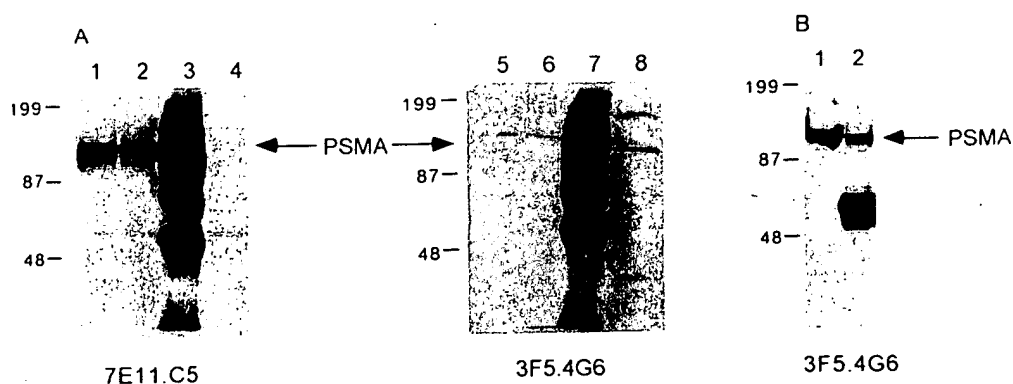
A

7E11.C5

3F5.4G6

B

3F5.4G6

Fig. 3.   A: Demonstration that the 7E11.C5 and 3F5.4G6 monoclonal antibodies recognize identical proteins and that 3F5.4G6 recognizes an additional protein corresponding to PSM'. LNCaP lysate was initially immunoprecipitated with 7E11.C5 monoclonal antibody and the immunoprecipitated material separated on SDS gels and probed in a Western blot assay with either 7E11.C5 (lanes 1–4) or with 3F5.4G6 (lanes 5–8) monoclonal antibodies. Lanes 1, 5, crude LNCaP lysate (0.05 μg protein/well); lanes 2, 6, precleared LNCaP lysate (0.05 μg protein/well); lanes 3, 7, material that immunoprecipitated with 7E11.C5 monoclonal antibody; lanes 4, 8, proteins left in the previously immunoprecipitated LNCaP lysate. It should be noted that 7E11.C5 immunoprecipitated a protein of M_r 120 kDa that was recognized not only by 7E11.C5 (lane 3), but also by 3F5.4G6 (lane 7). It should also be noted that present in the supernatant after 7E11.C5 immunoprecipitation was

a protein recognized by 3F5.4G6 (lane 8), but not by 7E11.C5 (lane 4), and that corresponds to PSM'. Thus, 7E11.C5 does not recognize PSM' (i.e., the epitope of 7E11.C5 is amino acids 1–7 of PSMA while the epitope of 3F5.4G6 is amino acid 716–723) and therefore PSM' should remain in the lysate of a 7E11.C5 immunoprecipitated LNCaP lysate and subsequently recognized by 3F5.4G6. **B:** Demonstration that monoclonal antibody 7E11.C5 and monoclonal antibody 3F5.4G6 recognize identical proteins. PSMA from an LNCaP lysate was immunoprecipitated by monoclonal antibody 3F5.4G6, the proteins in the immunoprecipitate separated on a SDS gel, the proteins transferred to Immobilon P and probed in a Western blot with monoclonal antibody 7E11.C5. Lane 1, LNCaP lysate control; lane 2, 3F5.4G6 immunoprecipitation of 2.5 μg LNCaP lysate and Western blot with 7E11.C5 monoclonal antibody.

nized PSMA in the serum of prostate cancer patients (stage D2; Fig. 4), illustrating its utility in detection of PSMA in the serum of prostate cancer patients.

Studies of the expression of PSMA in the serum of both normal individuals and prostatic cancer patients has provided conflicting results at two laboratories [1–6]. Troyer et al. [5,6] reported evidence suggesting that the binding of 7E11.C5 to Western blots of serum specimens was nonspecific in that the peptide containing the antibody epitope (N1–19) was ineffective in inhibiting antibody binding to a protein species migrating in the same region as PSMA. These studies used differing conditions than was previously reported by this laboratory wherein serum expression was demonstrated [1–4]. The results presented in Figure 5 using the procedures we originally described indicate that 7E11.C5 binding to Western blots of both LNCaP cell lysates and human serum is specific and not due to nonspecific binding of secondary antibody. No labeling of any protein band corresponding to PSMA was detected in the absence of the 7E11.C5 antibody, even after extended exposure of the blot to X-ray film. This evidence strongly supports the presence of PSMA in human serum and is consistent with our earlier results demonstrating elevations in serum PSMA in prostatic cancer patients in clinical progression. In our results, we have also failed to demonstrate PSMA protein in normal human brain tissue, in contrast to a report by Troyer and colleagues, which has
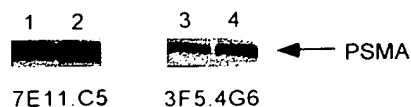

7E11.C5   3F5.4G6

Fig. 4. Demonstration by Western blot of PSMA in serum of prostate cancer patients (stage D2) using monoclonal antibody 7E11.C5 (lanes 1, 2) and demonstration of the recognition of PSMA in the same prostate cancer patient by monoclonal antibody3F5.4G6 (lanes 3, 4). 0.44μg serum protein was loaded in each well and protein separated on SDS gels as described in Materials and Methods.

the same methodological limitations noted in Figure 5 [5,6]. In addition, studies using the 9H10 monoclonal antibody have failed to show that it interacts with 3F5.4G6 or 7E11.C5 antigen [3].

Moreover, 3F5.4G6 reactivity was not detected in DU-145 or PC3 cell lines—only in LNCaP, as is the case with 7E11.C5 [3]. There is, however, a most recent report demonstrating a clear nucleotide sequence identity for a region of the extra-cellular portion of PSMA (i.e., in the PSM' distal region) which possesses properties of NAALADase membrane hydrolase (figure 2).[12] We are conducting further work to clarify the molecular characteristics of the NAALADase region to 3F5.4G6 and 7E11.C5. Nevertheless, the availability of the 3F5.4G6 antibody for application in a sandwich immunoassay will simplify the detection of serum PSMA and provide a potentially useful clinical tool for monitoring prostatic cancer patients.
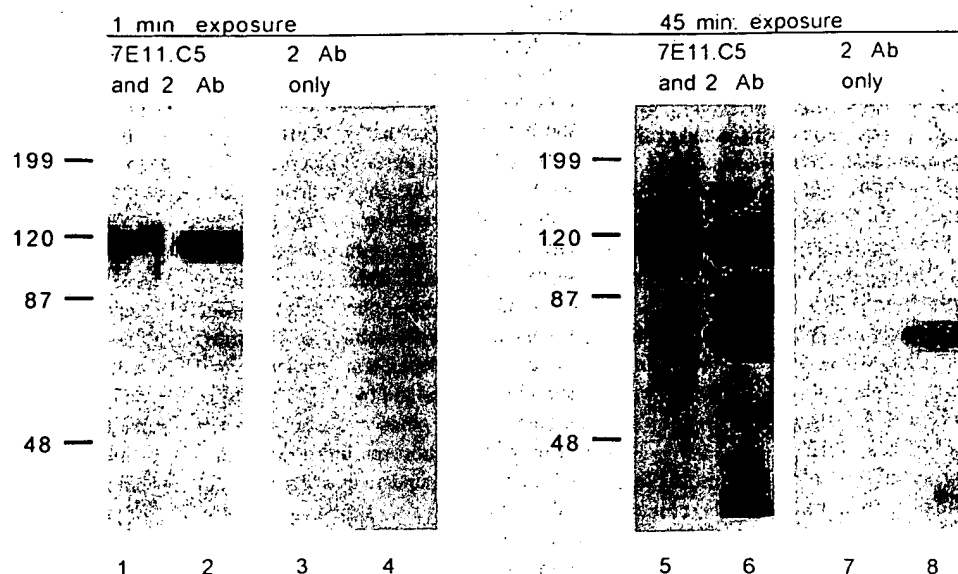
1 min exposure

7E11.C5	2 Ab
and 2 Ab	only

199 —

120 —

87 —

48 —

1   2   3   4

45 min. exposure

7E11.C5	2 Ab
and 2 Ab	only

199 —

120 —

87 —

48 —

5   6   7   8

Fig. 5. Demonstration that recognition of a protein in LNCaP lysate (lanes 1, 5) and in the serum of cancer patients (lanes 2, 6) by 7E11.C5 is specific to the primary antibody 7E11.C5 and not due to nonspecific recognition by secondary antibody used to report primary antibody binding to proteins. LNCaP lysate (0.05 μg protein/well; lanes 1,3,5,7) or prostate cancer patient serum (0.44 μg protein/well; lanes 2,4,6,8) was separated on SDS gels as previously described and transferred to Immobilon P paper and processed for Western blot as follows. Lanes 1,2,5,6 were probed with 7E11.C5 monoclonal antibody and then with secondary antibody (i.e., goat antimouse IgG) or with secondary antibody only (lanes 3,4,7,8). The film was exposed for either the routine 1 min (lanes 1–4) or overexposed for a period of 45 min (lanes 5–8). These results demonstrate that the recognition of bands by 7E11.C5 is specific for the primary antibody, and not due to nonspecific binding of secondary or reporting antibody. It should be noted that the same secondary antibody is used with monoclonal 3F5.4G6 as with 7E11.C5, and therefore the 3F5.4G6 monoclonal antibody is specific for PSMA and PSM' as illustrated in Figure 4, 6, 7 and is not due to nonspecific binding of the secondary antibody to proteins in the lysate.

ACKNOWLEDGMENT

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